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Investigation and *in vivo* validation of the anti-*Candida* activity of probiotic metabolites in the vaginal niche

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After a year filled with hard work, I am so happy and proud I can finally present my master thesis: “Investigation and *in vivo* validation of the anti-*Candida* activity of probiotic metabolites in the vaginal niche.” From the first moment I read the description of this master thesis topic until now, my interest in probiotics and vaginal fungal infections has only increased. Besides doing my thesis this year, I also got the opportunity to write a review together with my promotor Liesbeth Demuyser, Mart Sillen and Ilse Palmans named “The role of fatty acid metabolites in vaginal health and disease: application to candidiasis.” I can proudly say that it is already accepted and will be published in Frontiers Microbiology, section Infectious Diseases.

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ABBREVIATIONS

Als	Agglutinin-like sequence
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
C. albicans	*Candida albicans*
C. auris	*Candida auris*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
C. parapsilosis	*Candida parapsilosis*
C. tropicalis	*Candida tropicalis*
CFU	Colony forming units
Epa	Epithelial adhesin
FIC	Fractional inhibitory concentration
GPI	Glycosylphosphatidylinositol
HeLa	Henrietta Lacks cell line
Hwp	Hyphal wall protein
IL	Interleukin
MIC	Minimum inhibitory concentration
NADH	Nicotinamide adenine dinucleotide
OD600	Optical density at wavelength 600 nm
PBS	Phosphate-buffered saline
pH	Potential hydrogen
pKa	$-\log_{10}$ of acid dissociation constant
RNA	Ribonucleic acid
RVVC	Recurrent vulvovaginal candidiasis
S. cerevisiae	*Saccharomyces cerevisiae*
Sap	Secreted aspartic proteases
ScerViCs	*S. cerevisiae*-based treatment of vaginal candidiasis
SCFA	Short chain fatty acids
SEM	Standard error of the mean
UV	Ultra-violet
VSM	Vaginal simulative medium
VVC	Vulvovaginal candidiasis
WGD	Whole genome duplication
YPD	Yeast extract peptone dextrose
Yps	Yapsin secreted proteases

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SUMMARY

Vulvovaginal candidiasis (VVC) is a vaginal fungal infection caused by a *Candida* species. In most cases, VVC is caused by *Candida albicans* or *Candida glabrata*. Although 70% of all women worldwide suffer from it at least once in their lives, it is still a women's health condition that is severely under-reported and under-appreciated. The overuse of antifungal drugs is causing the development of increasingly more drug-resistant *Candida* species in patients. There is a clear need for alternatives to stop the development of a drug-resistance crisis in the future. The goal of this project was therefore to find a new probiotic treatment based on the yeast *Saccharomyces cerevisiae* to treat VVC and to reduce the use of antifungal drugs. Most existing probiotic treatments of VVC are based on lactobacilli, known for their lactate production, but they cannot be combined with antibiotic treatments, which are a major cause of VVC. *S. cerevisiae* can produce acetate and stimulate the production of propionate and butyrate in other microbiota. Therefore, the effects of short chain fatty acids (SCFA) acetate, propionate and butyrate were tested and compared with the effect of lactate on the growth of *C. albicans* and *C. glabrata.* Acetate, propionate and butyrate have an inhibitory effect on both *Candida* species while lactate stimulates their growth. It was tested whether this lack of inhibition might be due to the pH of the medium but the effect of lactate turns out to be not pH-dependent. There is also no difference between the effect of L- and D-lactate on *Candida* growth. These results suggest that the production of lactate is probably not the most important mechanism by which *Lactobacillus* species inhibit *Candida*. In the second part of this study, the azole susceptibility and synergy with acetate was tested. *C. glabrata* is more resistant to the antifungal drug fluconazole compared to *C. albicans* and there is no significant synergy between fluconazole and acetate. The last part of the study focused on the *in vivo* optimization of the mouse model for VVC. The selection of the right mouse substrain turned out to be very important. A direct injection of cell suspension results in a more stable vaginal *Candida* infection in mice compared to application with a cotton swab. Finally, using the optimized mouse model, a fluconazole-based treatment of VVC was compared with a *S. cerevisiae*-based probiotic treatment. The results were very promising. Two days after the probiotic treatment the vaginal load of *Candida* was lower than the untreated and fluconazole-treated group. The effect slowly diminished after a few days because the adhesion ability of the *S. cerevisiae* strain was probably not great. The inhibiting effect of fluconazole was less pronounced as expected. Together, these results point towards the potential of using probiotic *S. cerevisiae* strains in VVC therapy.

1. LITERATURE REVIEW

1.1. Clinical and economical relevance of vaginal infections

Vaginal fungal infections are very common. Of all women worldwide, 70% get a vaginal yeast infection at least once in their life, 40% get it more than once and 5% of all women worldwide suffer from the recurrent version and get at least four infections per year (Sobel, 2007). Denning *et al.* (2018) investigated the global burden of recurrent vaginal fungal infections. They stated that approximately 138 million women are affected by recurrent infections every year and that the economic cost of lost productivity in developed countries increases up to 11.84 billion euros. In these women, there is also a lot of resistance to existing drugs since they are treated frequently. A probiotic therapy that would help restore the balance in the microbiome could possibly be a solution for them.

1.2. Vaginal candidiasis

1.2.1. The vagina as a niche for infection

1.2.1.1. Anatomy and histology

The internal part of the female genital tract can be divided in three compartments: the lower genital tract, the endocervix and the upper genital tract (Figure 1B). The internal lower genital tract consists of the vagina, the ectocervix and the lower part of the endocervix (Cole, 2006). It used to be generally believed that the vulva and the lower genital tract are the only non-sterile parts of the female genital tract where microorganisms can grow and possibly cause an infection. The cervix is the lowest part of the uterus and produces a mucus that was believed to almost completely prevent microorganisms to move to the upper genital tract. However, Chen *et al.* (2017) demonstrated that this is not the case. They found microbial communities along the whole female genital tract, so also in the upper part (cervix, uterus and fallopian tubes). Intra-uterine *Candida* infections are possible but this research project focuses on vulvovaginal candidiasis (VVC) which are *Candida* infections in the lower genital tract since these occur much more frequent (Donders *et al.*, 1991). The largest part of the lower genital tract, the vagina, is a fibromuscular tube that is located within the pelvis posterior, anterior to the rectum and extends to the bladder. It connects the internal uterine cavity with the vulva (Figure 1A).

The vagina has three main functions: excretory channel for secretions of the uterus, sexual intercourse and childbirth (Female Genital Anatomy, 2002; Miranda, 2018). The diameter of the human vagina is in general about 2.5 cm but the tube is very distensible. It is most narrow at the vaginal opening and widest at the upper part (Dutta, 2014). Histologically, the vaginal wall is composed of three distinct layers: the internal mucosal layer, the intermediate muscularis layer and the external adventitial layer (Figure 1C). The mucosal layer consists of nonkeratinized squamous epithelium without glands and the lamina propria. The epithelium is hormone sensitive. It undergoes cyclical changes during the menstrual cycle such as keratinization and increased glycogen production. The surface of the mucosa is full of transverse ridges and bumps called rugae. The lamina propria underneath the epithelium is composed of loose connective tissue that contains many elastic fibers, giving the vagina its capability to distend. It also contains a dense network of blood vessels, lymphatic vessels and nerve fibers. The muscularis is composed of an outer longitudinal and inner circular muscle layer that contain autonomically innervated smooth muscle fibers. The third layer of the vagina, the adventitia, is rich in collagen, blood vessels, lymphatic vessels and nerves. The elasticity and toughness of the adventitia provides structural support and allows the expansion of the vagina during intercourse and childbirth (Dutta, 2014; Female Genital Anatomy, 2002; Miranda, 2018).

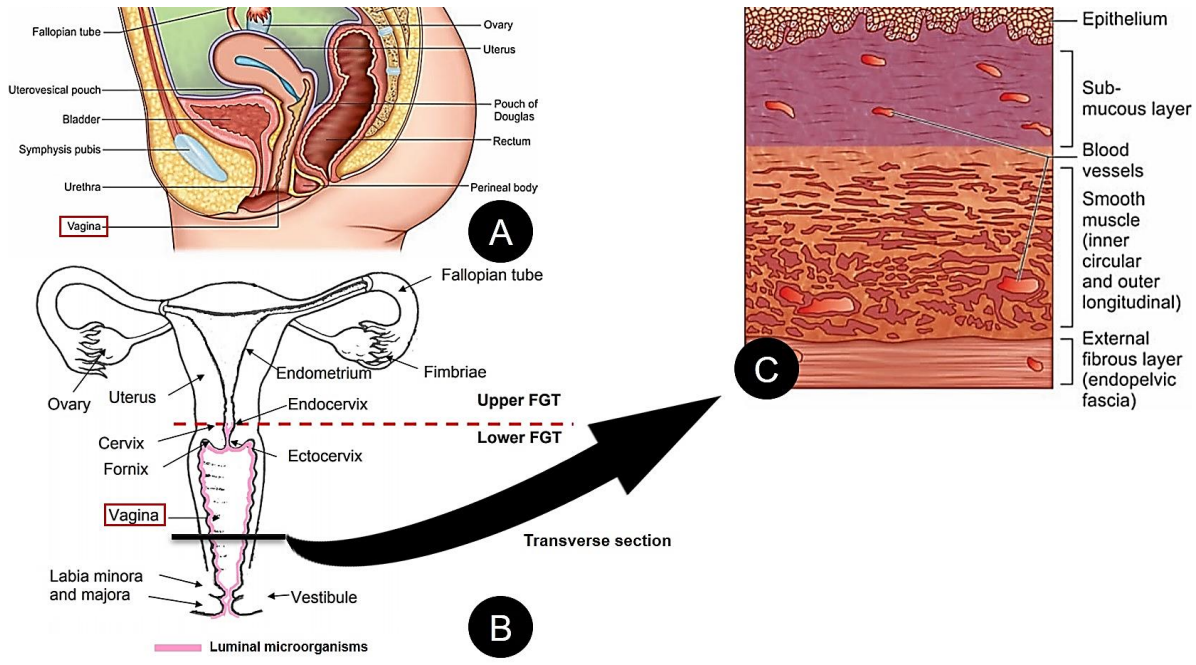


Figure 1: Anatomy and histology of the female genital tract.
(A) Mid-sagittal section of the female pelvis showing the location of the vagina in relation to other structures. (**B)** Organs of the female genital tract (FGT). **(C)** Transverse section of the vaginal wall. Modified from (Dutta, 2014; Nikolaitchouk, 2009).

1.2.1.2. pH

The pH of the human vagina is more acidic (3.8 - 4.8) compared to other mammals (5.4 - 7.8) (Miller *et al.*, 2016; O'Hanlon *et al.*, 2019). This is because the vaginal microbiota in most humans is composed for more than 70% of *Lactobacillus* bacteria. In other mammals, this bacterial species only constitutes less than 1% of their vaginal microbiome (Miller *et al.*, 2016). The regulation of the acidic vaginal pH in humans is also unique (Yildirim *et al.*, 2014). The human vagina is very nutrient-rich and thereby supports a diverse, dynamic microbiome (Farage & Maibach, 2006). This dynamicity seems to be caused by the constantly changing levels of the hormone estrogen throughout a woman's life. The reduction in vaginal lactobacilli after the menopause is mainly the result of the loss of estrogen (Willhite & O'Connell, 2001). The acidity of the human vaginal pH is maintained by three major mechanisms: the extracellular metabolism of glycogen by microorganisms, the intracellular metabolism of glycogen by intermediate cells and the proton secretion by epithelial cells (Godha *et al.*, 2018). The presence of estrogens and vaginal epithelial maturation create conditions that select for microorganisms like *Lactobacillus*, *Atopobium*, *Leptotrichia*, *Leuconostoc*, *Megasphaera*, *Pediococcus*, *Streptococcus* and *Weissella*. These organisms are capable of extracellularly fermenting glycogen and thereby producing lactate (Ravel *et al.*, 2011; Zhou *et al.*, 2004). Glycogen is produced by vaginal epithelial cells and is mainly deposited in the intermediate cells (Dominguez-Bello *et al.*, 2010). Throughout the menstrual cycle, the maturation of the vaginal epithelium and the production of glycogen is stimulated by estrogen (Godha *et al.*, 2018). This hormone regulates the viscosity and amount of vaginal secretion, the glycogen content of the epithelial cells and the oxygen-carbon dioxide levels (Hill *et al.*, 2005; Wagner & Ottesen, 1982). When the vaginal epithelium is shed, the microbiota will break down the glycogen-containing cells and eventually ferment the released glycogen (Nauth, 2007). The correlation between glycogen levels and the colonization and growth of *Lactobacillus* in the vagina has been recognized (Mirmonsef *et al.*, 2014). It has also been demonstrated in Spear *et al.* (2014) that an alpha amylase is present in the vagina that breaks down glycogen in simpler monosaccharides that can serve as nutrients for microorganisms. The second important contributor to the low vaginal pH is the anaerobic metabolism of the vaginal epithelium. In the vaginal epithelium there is a limited blood and oxygen supply. For that reason, the dominant metabolic mechanism in vaginal epithelial cells is anaerobic fermentation. The vaginal intermediate cells produce lactate as a byproduct through anaerobic fermentation of glycogen. This lactate (under the form of lactic acid, protonated) diffuses to the vaginal lumen and accumulates there (Freinkel, 2001). The last mechanism that contributes to the acidity of the vagina is the active proton excretion by vaginal superficial cells into the vaginal lumen. In the apical plasma membrane of vaginal epithelial cells, an estrogen-dependent V-type H^+-ATPase is present that actively pumps hydrogen ions into the vaginal lumen (Gorodeski, 2005; Gorodeski *et al.*, 2005).

1.2.1.3. Microbiome

In the squamous epithelium of the vaginal mucosa microbiota are present that play important roles in maintaining the health of the vagina. These microbial communities have several important functions like preserving the acidic vaginal pH, preventing the growth of pathogens, stimulating local immune responses and decreasing complications during pregnancies (Aldunate *et al.*, 2015; Boskey *et al.*, 2001; Delgado-Diaz *et al.*, 2019; Donders *et al.*, 2009; O'Hanlon *et al.*, 2019). Although the vaginal microbiome of every woman is unique, most are dominated by *Lactobacillus* bacteria. Archaea, protists, fungi and viruses can also be present in different concentrations. (Belay *et al.*, 1990; Bradford & Ravel, 2017; Drell *et al.*, 2013; Kusdian & Gould, 2014; Ravel *et al.*, 2011; Zhang *et al.*, 2021). In Ravel *et al.* (2011) the vaginal bacterial microbiota of 396 non-pregnant and asymptomatic North American women from four different ethnic groups (white, black, Asian and Hispanic) was analyzed. The bacterial communities clustered into five groups (I, II, III, IV and V). Four of these groups, found in 73% of all sampled women, were dominated by *Lactobacillus* species (I: *L. crispatus*; II: *L. gasseri*; III: *L. iners* and V: *L. jensenii*). The communities of the remaining 27% of women (group IV) were mainly characterized by obligate anaerobic bacteria including *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia* and *Mobiluncus*. Although fungi are extremely outnumbered by bacteria in the vaginal microbiome, they are important constituents of many but not all healthy vaginas. The percentage of asymptomatic women with fungal communities in their vagina is 20-50% (Barousse *et al.*, 2004; Drell *et al.*, 2013; Nowakowska *et al.*, 2004). The predominant and most detected species in vaginal samples is *C. albicans.* In Drell *et al.* (2013) the vaginal microbiomes of 494 asymptomatic, reproductive-age Estonian women were analyzed using next-generation sequencing. Of the vaginal samples that contained fungal communities and had a high enough sequence count (216 samples), 58% of sequences belonged to the Ascomycota phylum, 3% to Basidiomycota and the remaining sequences were unspecified. Various orders were found within Ascomycota: Saccharomycetales, Capnodiales, Eurotiales, Pleosporales and Helotiales. *Candida* species were detected in 70% of 216 samples. Of all sequences belonging to the *Candida* genus, 68% mapped to *C. albicans*. The composition of the vaginal microbiome can be very stable in some women while in others it can be very dynamic (Barrientos-Duran *et al.*, 2020). Factors that influence the microbial composition are steroid sex hormone levels, pregnancy, ethnicity, tobacco, drugs, stress, sexual activity, diet, hygiene, *etc*. (Barrientos-Duran *et al.*, 2020; Laghi *et al.*, 2014; Nansel *et al.*, 2006; Nelson *et al.*, 2018; Noyes *et al.*, 2018; Thoma *et al.*, 2011).

1.2.1.4. Vaginal fluid

The vaginal epithelium is kept moist by a vaginal fluid which leaks out of the vagina and is known as vaginal discharge. Depending on its consistency, color and smell different vaginal infections can be recognized (Spence & Melville, 2007). The vaginal fluid is made up of plasma transudate that seeps through the vaginal epithelium, secretions from the vaginal epithelial cells, Bartholin glands and Skene glands, residual urine, cervical and endometrial fluids and metabolites produced by vaginal microorganisms. Women always have approximately 0.5 – 0.75 g of vaginal fluid present in their vagina and produce in total about 6 g per day. The vaginal fluid primarily contains salts, proteins, fatty acids, and carbohydrates of which the concentrations can vary depending on the technique used to measure them (Owen & Katz, 1999). The amount of salts in the vaginal fluid without sexual stimulation lies around 1.38 g/L for sodium, 0.987 g/L for potassium, 0.120 g/L for calcium and 2.13 g/L for chloride (Levin & Wagner, 1977; Mende *et al.*, 1990; Stamey & Timothy, 1975; Wagner & Levin, 1980). The total protein content of the vaginal fluid ranges between 0.015 – 0.026 g/L (Raffi *et al.*, 1977). The concentration of lactate and acetate ranges between 1 – 5 g/L and 0.2 – 0.52 g/L respectively (Huggins & Preti, 1976; Oberst & Plass, 1936; Preti *et al.*, 1979). Glycogen is present in a range from 0.1 – 32 g/L and glucose from <0.1 – 13.3 g/L (Mirmonsef *et al.*, 2016). Glycerol and urea can also be found in the vaginal fluid in concentrations of 0.16 g/L and 0.3 – 0.49 g/L respectively (Huggins & Preti, 1976; Preti *et al.*, 1979; Wagner & Levin, 1978). The composition of the vaginal simulative medium (VSM) that is used in this study is based on previous concentrations (Owen & Katz, 1999). Most metabolites produced by vaginal microbiota are part of the following classes: amines, organic acids, short chain fatty acids (SCFA), amino acids, nitrogenous bases and monosaccharides (Ceccarani *et al.*, 2019; Vitali *et al.*, 2015). The concentration of many of these metabolites changes significantly in women when they have a vaginal infection. Ceccarani *et al.* (2019) found that in women with VVC, levels of trimethylamine N-oxide, taurine, methanol, isopropanol, O-acetylcholine and glucose were significantly increased compared to healthy women, while concentrations of lactate, 4-hydroxyphenylacetate, phenylalanine, pi-methylhistidine, glycine, dimethylamine and sarcosine were significantly lower.

1.2.2. Vaginitis

A lot of different factors can influence the concentrations of microorganisms in the vagina. When the microbial composition changes drastically, vaginal dysbiosis can occur. In most cases, this is characterized by low concentrations of *Lactobacillus* species and an increase of anaerobic microorganisms (Tachedjian *et al.*, 2017; Vitali *et al.*, 2015). Vaginal dysbiosis can eventually lead to vaginitis, which is inflammation of the vagina. Depending on what pathogenic organism dominates, different types of vaginal infections can be distinguished. The three most common causes of vaginitis are bacterial vaginosis, VVC and trichomoniasis. Bacterial vaginosis is diagnosed in 40-50% of women with vaginitis. It is a polymicrobial infection caused by an overgrowth of anaerobic bacteria like *Prevotella*, *Mobiluncus*, *Gardnerella vaginalis*, *Ureaplasma* and *Mycoplasma*. The symptoms of bacterial vaginosis are a fishy odour and a thin, greyish, homogenous vaginal discharge. Burning and itching of the vulva are typically absent. VVC is diagnosed in 20-25% of women with vaginitis (Mills, 2017; Paladine & Desai, 2018). As the name suggests, this type of infections is caused by *Candida* species. In most women with VVC, *C. albicans* is identified. In epidemiological studies done before 2001, 85-95% of all VVC cases are caused by *C. albicans* (Linhares *et al.*, 2001; Lynch & Sobel, 1994; Otero *et al.*, 1998; Saporiti *et al.*, 2001; Spinillo *et al.*, 1997). In more recent studies, the percentage of vaginal infections caused by *C. albicans* ranges between 35-90%, *C. glabrata* between 3-50%, *C. tropicalis* between 0-18%, *C. parapsilosis* between 0-10% and *C. krusei* between 0-6% depending on the country where the study was conducted (Ahmad & Khan, 2009; Cetin *et al.*, 2007; Fan *et al.*, 2008; Gonçalves *et al.*, 2016; Mohanty *et al.*, 2007; Okungbowa *et al.*, 2003; Paulitsch *et al.*, 2006). The increase of non-*albicans Candida* infections could be the result of the inappropriate use of antifungal drugs. Non-*albicans Candida* species are more resistant to the most used antifungal treatments than *C. albicans* (Richter *et al.*, 2005). The overuse of these treatments could lead to the selection of more resistant *Candida* species (Gonçalves *et al.*, 2016). Non-*albicans Candida* species are also more often found in women affected by recurrent vulvovaginal candidiasis (RVVC) (Amouri *et al.*, 2011; Grigoriou *et al.*, 2006; Richter *et al.*, 2005). Patients suffering from RVVC get at least four infections each year so they are treated very regularly which speeds up the selection process (Gonçalves *et al.*, 2016). The symptoms of a vaginal fungal infection are a thick, white vaginal discharge and itching and burning of the vulva. VVC and especially RVVC severely impair the wellbeing, and quality of life. Also, they are typically associated with mental distress, low self-esteem, physical pain and sexual dysfunction. In addition, there are reports that indicate an involvement in late miscarriage, preterm labor, infertility and pelvic inflammatory disease (Gonçalves *et al.*, 2016). Trichomoniasis is diagnosed in 15-20% of women with vaginitis (Mills, 2017; Paladine & Desai, 2018). It is a sexually transmitted infection caused by the anaerobic parasitic protozoan *Trichomonas vaginalis*.

The symptoms of trichomoniasis are a green/yellow vaginal discharge, itching of the vulva, vaginal pain and a bad odour. Women with trichomoniasis are also two to three times more likely to acquire human immunodeficiency virus (HIV) (Kissinger, 2015).

1.2.3. *Candida* species causing vaginal infections

1.2.3.1. Taxonomy & phylogeny

The name "*Candida*" is derived from the Latin word "candidus", which means "glowing white." This term was chosen because of the creamy glistening color that *Candida* colonies have (Hameed *et al.*, 2018). The genus *Candida* belongs to Superkingdom: Eukaryota, Kingdom: Fungi, Phylum: Ascomycota, Subphylum: Saccharomycotina, Class: Saccharomycetes and Order: Saccharomycetales (McManus & Coleman, 2014). *Candida* includes around 150 species of which about 30 species are capable of infecting humans (Hameed *et al.*, 2018; McManus & Coleman, 2014). Not all *Candida* species share the same evolutionary origin so they are spread over several clades (Figure 2). This is because during classification the term "*Candida*" was given to all imperfect fungi with no clearly defined sexual cycle (Turner & Butler, 2014). Most pathogenic *Candida* species like *C. albicans*, *C. parapsilosis*, *C. tropicalis* and the more recently discovered invasive *C. auris* do belong to the same clade: the CTG clade. It is part of the Saccharomycetales incertae sedis family within the order Saccharomycetales (McManus & Coleman, 2014). The species in the CTG clade translate the CUG codon, typically translated as leucine, as serine (Santos *et al.*, 1993). This substitution is not complete, it is estimated that still 3% of CUG codons are translated as the conventional leucine (Suzuki *et al.*, 1997). The second clade that contains important pathogenic *Candida* species is the genus *Nakaseomyces*. It belongs to the Saccharomycetaceae family, that also contains the yeast *S. cerevisiae*, within the order Saccharomycetales (Kurtzman, 2003). *Nakaseomyces* is one of the WGD-clades, that are characterized by whole-genome duplication (Wolfe & Shields, 1997). *C. glabrata* is the best-known pathogenic species from this clade. *C. bracarensis* and *C. nivarensis* are two more recently identified pathogens of the *Nakaseomyces* lineage with a growing incidence (Gabaldón & Carreté, 2016). The only pathogenic *Candida* species that is not part of the CTG or *Nakaseomyces* clade is *C. krusei*. Because *Candida* species are dispersed over different clades there is also phenotypical diversity between them. *C. albicans* and *C. glabrata* are the two most common pathogenic *Candida* species but even though they share the same genus name, they are only distantly related. *C. glabrata* is even more closely related to *S. cerevisiae* than to *C. albicans* (Brunke & Hube, 2013). Due to new developments in fungal taxonomy and increased knowledge about genomic data analysis many *Candida* species are already renamed and the *Candida* genus will probably be completely redefined in the next years (Brandt & Lockhart, 2012; Hawksworth, 2012).

For the time being, pathogenic *Candida* species should be looked at as a collection of diverse infectious fungi in which, despite their shared genus name and common medical term “candidiasis” for their caused infections, quite some phenotypic diversity is present (Gabaldón *et al.*, 2016).

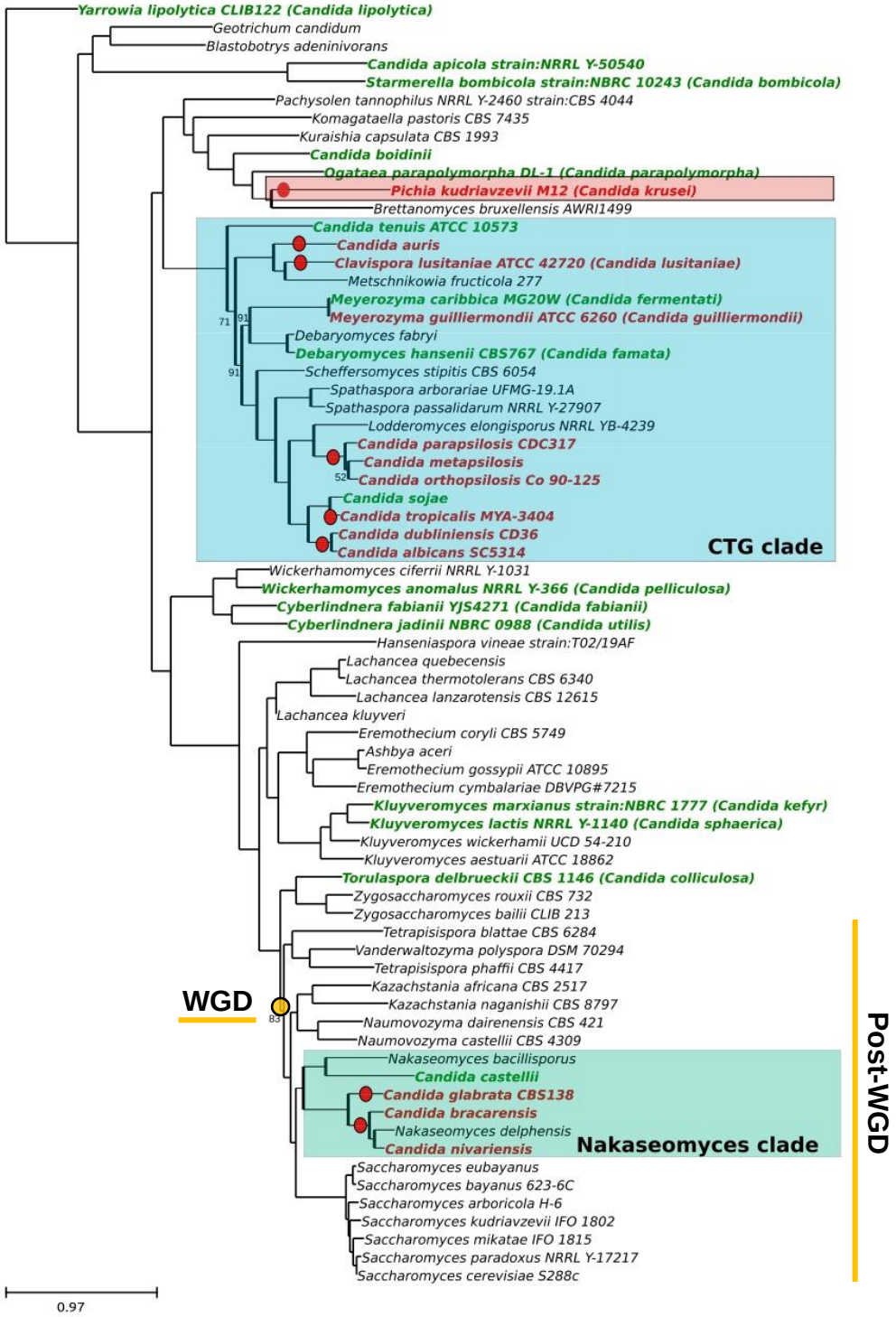


Figure 2: Phylogenetic relationships between *Candida* species.
Species names in red indicate pathogenic species and species names in green indicate non-pathogenic species. The CTG clade, *Nakaseomyces* clade and post-WGD clades are indicated. The scale represents bootstrap values. Modified from (Gabaldón *et al.*, 2016; Mell & Burgess, 2003).

1.2.3.2. Virulence factors relevant for vaginal infections

As previously stated, *C. albicans* and *C. glabrata* are the two main causative species of vaginal *Candida* infections. For this reason, the focus of this part will specifically lie on the virulence factors of these species. An important difference to begin with is that *C. albicans* is diploid while *C. glabrata* is a strictly haploid fungus (Kaur *et al.*, 2005). Because of the phylogenetic and phenotypic differences between both, they rely on somewhat different strategies to survive in the host. They share the same basic concepts, but the outcome is different. The strategy of *C. albicans* is to actively subvert the immune system of the host and obtain nutrients. Its strategy is way more aggressive than the strategy of *C. glabrata*, which is focused on stealth, persistence and evasion to stay as unnoticed as possible (Brunke & Hube, 2013).

A) Adhesion and invasion

To establish a vaginal infection, *Candida* species need to be able to adhere to vaginal epithelial cells of the host. Both *Candida* species have independently evolved specialized proteins, adhesins, to accomplish the adhesion to not only host cells but also other *Candida* cells, other microorganisms and abiotic surfaces like medical devices (Garcia *et al.*, 2011; Verstrepen & Klis, 2006). These adhesins are expressed on the surface of their cells upon detection of certain cues of the host (Brunke & Hube, 2013). The best known adhesins in *C. albicans* are agglutinin-like sequence (ALS) proteins and hyphal wall protein 1 (Hwp1). *ALS* genes encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins and Hwp1 is a hypha-associated glycosylphosphatidylinositol-linked protein (Murciano *et al.*, 2012; Phan *et al.*, 2007; Staab *et al.*, 1999). Mammalian transglutaminases can use Hwp1 as a substrate which results in a covalent binding between *C. albicans* hyphae and host cells (Staab *et al.*, 1999). To invade host cells, *C. albicans* uses two different methods: fungal-induced endocytosis and active penetration (Mayer *et al.*, 2013; Naglik *et al.*, 2011; Zakikhany *et al.*, 2007). During induced endocytosis special fungal cell surface proteins, invasins, bind to receptors on the surface of the host cells, such as E-cadherin (Phan *et al.*, 2007). This binding triggers the epithelial cells to engulf the fungal cells. Induced endocytosis is a passive process because even dead hyphae can be endocytosed (Park *et al.*, 2005). Until now only two invasins are identified in *C. albicans*, Als3, which is also an important adhesin, and Ssa1 (Phan *et al.*, 2007; Sun *et al.*, 2010). Ssa1 is one of the two only members of the heat shock protein 70 family in *C. albicans*. It is expressed on the cell surface of both yeast cells and hyphae (Sun *et al.*, 2010). In contrast, as the name suggests active penetration is an active process so it requires living *C. albicans* hyphae (Dalle *et al.*, 2010; Wächtler *et al.*, 2011). During active penetration, the hyphal cells probably use a combination of physical pressure, hydrolytic enzymes and other unknown factors to invade the host cells. The exact molecular mechanisms of active penetration are still unknown (Wächtler *et al.*, 2012).

The main adhesins in *C. glabrata* that are important for adhesion are the Epa (Epithelial Adhesin) proteins (Cormack *et al.*, 1999). Just like *ALS* genes in *C. albicans*, *EPA* genes also encode GPI-linked cell wall proteins. Epa1, Epa,6 and Epa7 are the most important adhesins for adhesion to epithelial surfaces. In mice, it has been demonstrated that Epa1 is specifically important for adhesion to uroepithelial cells (Achkar & Fries, 2010). *C. glabrata* only grows in yeast form. Since it is not able to form hyphae, invasion via active penetration is not possible. Although it is known that *C. glabrata* can invade deeper tissues, the exact mode of invasion is not known yet. When the epithelial barrier is disrupted, by for example *C. albicans* hyphae, *C. glabrata* can easily invade. When these barrier disruptions are absent, it is suggested that *C. glabrata* may also use epithelial endocytosis like *C. albicans* for invasion (Brunke & Hube, 2013).

B) Morphological and phenotypic plasticity

One of the most important virulence factors of *C. albicans* is its morphological plasticity. It can grow as unicellular oval-shaped yeast cells, filamentous hyphae and pseudohyphae (Gow *et al.*, 2002). Hyphae are chains of elongated cells divided by septa and pseudohyphae are chains of elongated cells that lack septa but have contractions at the cell-cell junctions between cells (Merson-Davies & Odds, 1989; Sudbery, 2001). *C. albicans* can switch between these morphological forms to maximize the benefits of each form during different phases of infection (Jacobsen *et al.*, 2012). Strains that lose the ability to switch between different morphological forms are less pathogenic and have a reduced colonization capacity (Lo *et al.*, 1997). Yeast cells are important in the beginning of an infection because filamentation can only start after adhesion to host cells (Tang *et al.*, 2016). In general, the yeast form has been shown to be less invasive than the hyphal form (Berman & Sudbery, 2002). Nevertheless, the yeast form is the primary form in dissemination through the bloodstream which can cause serious systemic infections (Saville *et al.*, 2003). Hyphae start the invasion process by elongating, stretching and eventually penetrating the host cells (Dalle *et al.*, 2010). They are harder to kill by the immune system of the host because of their larger size and their capability of killing macrophages by penetration (McKenzie *et al.*, 2010). The functions of pseudohyphae are currently unknown but it is clear that they will differ from the functions of hyphae since there are remarkable differences between not only the morphology and structure of pseudohyphae and hyphae but also the metabolism and mechanism of cell division (Mayer *et al.*, 2013; Sudbery *et al.*, 2004). It will be very interesting to find out what the exact role of pseudohyphae in an infection is since Roselletti *et al.* (2019) suggests that pseudohyphae rather than true hyphae are critical in VVC. In addition to the yeast-hyphae switch, *C. albicans* can also undergo another type of phenotypic transformation: the white-opaque switch. White cells are round and form bright, dome-shaped colonies, while opaque cells are more elongated and form darker, flatter colonies (Slutsky *et al.*, 1987).

The two phenotypic forms also differ in their gene expression, nutritional resources, preferred niches and signals to induce virulence (Calderone & Fonzi, 2001). Switching from white-state to opaque-state only occurs in cells of the a- and α-mating type but not in diploid a/α cells. Mating in *C. albicans* mainly occurs between opaque cells because it is a million times more efficient than mating between white cells (Miller & Johnson, 2002).

As previously stated, *C. glabrata* is strictly haploid and unable to form hyphae. So, in contrast to *C. albicans*, morphological flexibility is not important for its pathogenicity (Brunke & Hube, 2013). Although *C. glabrata* cells can be found in two mating types (a and α), mating has never been documented in this species. Also, *C. glabrata* is only rarely observed to switch from one mating type to the other because efficient switching is correlated with a high mortality rate (Boisnard *et al.*, 2015). Another morphological difference between the two species is the size of the fungal cells. *C. glabrata* cells are on average 1-4 µm, while *C. albicans* cells are on average 4-6 µm (Larone, 2002).

C) Secretion of hydrolytic enzymes

After adhesion to host cells, *C. albicans* yeast cells can switch to hyphal cells to start the invasion process. *C. albicans* is capable of producing four different families of hydrolytic enzymes to help adhere to and penetrate the host cells: proteases, phospholipases, lipases and haemolysins (Silva *et al.*, 2012). Besides facilitating the penetration of the host cells, these enzymes also help with nutrient acquisition by digesting molecules (Naglik *et al.*, 2003; Wächtler *et al.*, 2012). The first family contains ten secreted aspartic proteases (Saps). Eight of ten Saps are actually secreted (Sap1-8), while Sap9 and Sap10 remain bound to the surface of the fungal cell (Monod *et al.*, 1998; Naglik *et al.*, 2003). Aspartic proteases cleave peptides by using a water molecule, bound to an aspartic acid at the active site of the protease, to do a nucleophilic attack on the peptide bond that needs to be cleaved (Mandujano-González *et al.*, 2016). Saps are unique as proteases in the way that they perform best in acidic environments (pH 3-4) (Rao *et al.*, 1998). This is an important factor in VVC since the vaginal pH lies around 4 (O'Hanlon *et al.*, 2019). It has been shown that there is a strong correlation between the expression of Sap1-3 and VVC (Lian & Liu, 2007). Sap2 is an example of a protease with a very broad substrate specificity. It can cleave a wide variety of human proteins such as mucin, endothelial cell proteins, coagulation factors, clotting factors, immune system molecules like immunoglobulin A and extracellular matrix proteins like keratin, collagen and vimentin (Naglik *et al.*, 2003). The second family of hydrolytic enzymes of *C. albicans* contains four classes of phospholipases (A, B, C and D) (Niewerth & Korting, 2001). Phospholipases disrupt the membranes of host cells and help yeast cells adhere to host cells by hydrolyzing ester bonds in the glycerophospholipids of host cell membranes (Ghannoum, 2000).

In *C. albicans*, seven different phospholipases have been identified: PLA, PLB1-2, PLC1-3 and PLD1 (Samaranayake *et al.*, 2006). The third family consists of ten lipases (Lip1-10) which are capable of hydrolyzing triacylglycerol (Hube *et al.*, 2000). These lipases contribute to adhesion, cell damage and can influence the host immune system (Stehr *et al.*, 2004). The last family contains haemolysins, which degrade hemoglobin and cause the lysis of red blood cells. This is essential for *C. albicans* to recover iron and survive in the host. The genes and mechanism responsible for haemolytic activity are not fully elucidated yet (Gonçalves *et al.*, 2016; Manns *et al.*, 1994).

C. glabrata also produces hydrolytic enzymes: Yapsin Secreted Proteases (Yps). These enzymes are GPI-linked aspartyl proteases and have again many similarities with the Saps of *C. albicans* (Oliver *et al.*, 2019). Yps enzymes are essential for the virulence of *C. glabrata* as they play an important role in its survival strategy that mainly relies on evasion of the immune system. Yps enzymes are important for intracellular survival and proliferation in macrophages, cell wall homeostasis and in the processing of GPI-linked adhesins like Epa1 (Kaur *et al.*, 2007). They suppress interleukin-1 beta (IL-1β) production in activated macrophages. This is very important in evading the immune system since IL-1β is a key mediator during the inflammatory response of the host (Rasheed *et al.*, 2018). More strategies that *C. glabrata* uses to extend and ensure survival in the host are preventing the maturation of phagosomes, replicating within phagosomes till they burst and neutralizing oxidative bursts (Brunke & Hube, 2013).

D) Biofilm formation

Another important virulence factor of *C. albicans* is that in addition to growing as individual planktonic cells, it is also capable of forming a biofilm on both biotic and abiotic surfaces (Fanning & Mitchell, 2012). A biofilm is a highly organized community of fungal cells that is irreversible attached to a surface and produces a protective extracellular matrix (Douglas, 2003). Biofilm formation is a sequential process that consists of three phases: the early phase, intermediate phase and maturation phase. In the early phase, yeast cells adhere to a surface, start to proliferate and form a discrete colony. In the intermediate phase, the cells become more organized, filamentation starts in the upper part of the forming biofilm and secretion of extracellular polymeric substances (EPS) starts. These EPS and the proliferating cells will develop in a robust pluricellular tridimensional community, that we call a mature biofilm (Cavalheiro & Teixeira, 2018). The mature biofilm of *C. albicans* consists of two layers, a layer of blastopores with on top of that a layer of hyphae, surrounded by a thick matrix (Chandra *et al.*, 2001). After formation of a mature biofilm, yeast cells of this biofilm can detach, migrate to other niches and start a new biofilm there (Cavalheiro & Teixeira, 2018).

Forming a biofilm has several advantages. First of all, cells in a biofilm are more protected against environmental factors compared to planktonic cells. There is an increased resistance to antifungal drugs, defence mechanisms of the host and other chemical and physical stresses (Donlan, 2002). Other benefits are increased nutrient availability, metabolic cooperation and more chance of genetic exchange (Mohandas & Ballal, 2011). It has been reported that *C. albicans* can form biofilms *in vivo* on vaginal epithelium of mice and on intrauterine devices that women use as contraceptive, which are known to promote VVC (Chassot *et al.*, 2008; Harriott *et al.*, 2010; Lal *et al.*, 2008).

Just like *C. albicans*, *C. glabrata* is also capable of forming biofilms. Epa6 and Epa7 are important adhesins during biofilm formation and were also found to be essential during infections of the urinary tract. The biofilms of *C. glabrata* consist of a monolayer or multilayer of yeast cells (Ciurea *et al.*, 2020). Although *C. glabrata* does not form biofilms on vaginal epithelium in mice, it is able to form biofilms on abiotic surfaces like for example urinary catheters (Zangl *et al.*, 2019).

The increased resistance of biofilms against conventional antifungal therapies may be an explanation for the frequent recurrence of VVC (Gonçalves *et al.*, 2016). However, up until now the presence of biofilms in human VVC has not yet been successfully demonstrated in a clinical study. Thus, whether biofilms play an important role in human VVC cannot be said with certainty yet.

E) Drug tolerance

An important virulence factor that is specific for *C. glabrata* is its drug tolerance. This species has a lower intrinsic susceptibility to current available antifungal treatments, especially azoles, compared to *C. albicans* (Roetzer *et al.*, 2011). In addition to this, *C. glabrata* more frequently develops a high resistance after exposure to an azole treatment. There are several mechanisms by which *C. glabrata* can develop resistance. The most important one is the upregulation of drug efflux pumps. Many clinical isolates have activating mutations in transcription factor gene *PDR1*. Pdr1 binds to the response elements of ATP Binding Cassette (ABC) genes *CDR1*, *CDR2*, *SNQ2*, which encode efflux pump proteins. This results in an upregulation of their expression and an increased efflux of drugs. *C. glabrata* can also grow with altered sterols in their cell membrane by which it evades the action of azoles (Whaley *et al.*, 2017). The mode of action of azoles and other antifungal drugs is described in 1.3. The number of clinical *C. glabrata* isolates, also from women with VVC, that turn out to be multidrug resistant is very concerning (Whaley *et al.*, 2017; Yassin *et al.*, 2020). Nakamura-Vasconcelos *et al.* (2017) found that azole-resistant *C. glabrata* isolates adhere more efficiently and are better able to form biofilms in the vagina compared to not-resistant isolates.

Isolates of both *C. albicans* and *C. glabrata* from women with RVVC show a lower drug susceptibility than from women with VVC probably due to selection. Studies have demonstrated that most women with azole resistant *Candida* species previously used azoles (Lírio *et al.*, 2019). This all shows how important it is that infections are diagnosed well and that the general use of antifungal drugs is reduced, by for example alternate use of probiotics, to prevent the development of drug resistant species and possibly RVVC.

1.3. Current treatments of (vaginal) *Candida* infections

There are various antifungal agents available for *Candida* infections such as azoles, polyenes, echinocandins, *etc*. Azoles, specifically fluconazole treatments, are the most preferred and frequent used of all. Azoles target the ergosterol synthesis in the endoplasmic reticulum of the fungal cell and thus carry out their action from within the fungal cell. They inhibit the enzyme lanosterol 14-α-demethylase that transforms lanosterol into the fungal specific sterol, ergosterol. Azoles will only inhibit the growth of *Candida* cells and not kill them. They are therefore called fungistatic agents (de Oliveira Santos *et al.*, 2018). Polyenes, such as amphotericin B, bind to ergosterol in the fungal membrane and thereby disrupt the structure of the membrane. This causes extravasation of intracellular components which will lead to cell death (de Oliveira Santos *et al.*, 2018). Echinocandins, like for example caspofungin, target glucan synthesis in the fungal cell wall by inhibiting β-D-glucan synthase. By decreasing glucan levels, the fungal cell wall becomes weak and no longer resistant to osmotic stress. This will lead to fungal cell lysis. So, both polyenes and echinocandins are fungicidal agents (de Oliveira Santos *et al.*, 2018). This month, for the first time in 20 years, a drug came out in a completely new antifungal drug class: Ibrexafungerp. This drug class has a similar mechanism of action as echinocandins by targeting glucan synthesis in the fungal cell wall but with the major advantages that it has an increased activity at low pH and can be administered orally. Besides azoles, it is the only oral treatment for vaginal yeast infections on the market (Jallow & Govender, 2021). Depending on the site of infection, the causative *Candida* species and the severeness of the infection, different antifungal agents are recommended (de Oliveira Santos *et al.*, 2018). The current treatment recommendations for VVC caused by *C. albicans* are topical azole formulations, usually miconazole, or a single oral dose of fluconazole (Pappas *et al.*, 2016). For more severe, acute VVC, multiple doses of fluconazole are administered. However, these treatment recommendations could change in the near future with the approval of Ibrexafungerp. To treat a *C. glabrata* infection, a topical therapy of boric acid is combined with amphotericin B because *C. glabrata* is resistant to azoles. RVVC is treated by daily oral administration of fluconazole, after which therapy is continued weekly for six months. It is noteworthy, however, that 40 to 50% of women treated for RVVC will experience recolonization with *Candida* within 30 days after therapy cessation (Sobel *et al.*, 2001). Furthermore, as previously stated, a prolonged treatment can cause resistance (Marchaim *et al.*, 2012).

For the treatment of RVVC a ReCiDiF (Recurrent Candida infections treated with Degressive individualized doses of Fluconazole) approach is recommended where personalized reduction of the azole treatment is applied (Donders *et al.*, 2008). This approach allows to find the lowest drug dose to remain symptom-free.

1.4. Using probiotic therapy against *Candida* infections

Probiotics are live organisms that can have a beneficial effect on the user, if they are administered in sufficient amounts (Reid *et al.*, 2003). At this moment, most well-characterized and used microorganisms as probiotics are bacterial species of the genera *Lactobacillus* and *Bifidobacteria* (Bermudez-Brito *et al.*, 2012).

1.4.1. Lactobacilli

As previously stated, *Lactobacillus* species make up the biggest part of the vaginal microbiome of most women. They are the major producers of vaginal metabolites with as main end product lactate (Redondo-Lopez *et al.*, 1990). The rate of lactate production is strain specific. Lactobacilli have been shown to inhibit *Candida* species by inhibiting adhesion to epithelial cells, filamentation and biofilm formation. The mechanisms by which they exert their anti-*Candida* effect is strain dependent. Some examples of these mechanisms are the production of acids, such as lactate, biosurfactants, hydrogen peroxide and coaggregation molecules, and stimulation of the immune system of the host (Zangl *et al.*, 2019). However, there is still controversy whether lactate, produced by lactobacilli, is actually capable of inhibiting *Candida* growth at low pH due to contradictory results coming from various studies (Kasper *et al.*, 2015; Krasner *et al.*, 1956). Although there is uncertainty about the role of lactate in the antifungal effect of lactobacillus species, there are numerous other mechanisms by which they can inhibit *Candida*. Their natural dominance in the vaginal microbiome together with the promising results of various *in vitro*, *in vivo* and clinical studies explain the major attention these bacterial species get in the development of vaginal probiotics.

1.4.2. Fungal probiotics

Besides bacterial probiotics, the interest in fungi-based probiotics is also growing the last couple of years (Bermudez-Brito *et al.*, 2012). This is not surprising since they offer important advantages over bacterial probiotics. Fungi have a unique cell wall construction. It consists of two layers of which the inner layer is composed of chitin, 1,3-β-glucan and 1,6-β-glucan and the outer layer contains mannan (Lipke & Ovalle, 1998). This structure allows fungi to easily survive passage through the gastrointestinal tract as many probiotics are taken orally (Banik *et al.*, 2019).

Moreover, fungal probiotics are resistant to antibiotics. This means that, in addition to the fact that their antibiotic resistance profile does not need to be investigated, they can also be used in patients taking antibiotics, which is an important risk factor for VVC (Gaziano *et al.*, 2020; Xu *et al.*, 2008). The most common fungal probiotic on the market is *Saccharomyces cerevisiae* var. *boulardii* (Sen & Mansell, 2020). This yeast is currently being used in the treatment of chronic and acute gastrointestinal diseases like inflammatory bowel disease, bacterial and rotaviral diarrhea (Kelesidis & Pothoulakis, 2012; Madsen, 2001). Only recently, it has been demonstrated that *S. cerevisiae*-based probiotics show potential for treatment of not only VVC, but also bacterial vaginosis (Cayzeele-Decherf *et al.*, 2017; Gabrielli *et al.*, 2018; Pericolini *et al.*, 2017; Sabbatini *et al.*, 2018).

1.4.2.1. Lifecycle and growth conditions of *S. cerevisiae*

S. cerevisiae is a budding yeast that is one of the best studied and most widely used eukaryotes not only as model organism in scientific research but also as probiotic and for industrial purposes such as the production of wine, beer, vodka, bread, chocolate, bioethanol, *etc*. (Gaziano *et al.*, 2020; Mell & Burgess, 2003; Palma *et al.*, 2015; Parapouli *et al.*, 2020). The lifecycle of *S. cerevisiae* is very similar to the general lifecycle of any eukaryotic organism. They alternate between two states: a haploid state (1N), in which they contain one set of chromosomes (16 chromosomes), and a diploid state (2N), in which they contain two sets of chromosomes (32 chromosomes). As the name “budding yeast” already suggests, *S. cerevisiae* cells can reproduce asexually by budding. A bud forms on the mother cell, which will become a genetically identical copy of the mother cell, called a daughter cell. *S. cerevisiae* also has a sexual cycle, in which haploid cells mate to form diploid cells. This mating can only happen between two different mating types: a and α. Diploid yeast cells can either divide mitotically by budding or they can form four haploid spores (two of each mating type) by meiosis (Mell & Burgess, 2003).

For *S. cerevisiae* to perform its probiotic actions, it is very important that the growth conditions of the vaginal niche correspond to the growth range and needs of the probiotic yeast. *S. cerevisiae* strains can grow between temperatures of 1°C to 45°C with an optimal growth temperature of 30°C so the body temperature of humans (37°C) falls perfectly in this growth range. It has also been demonstrated in studies that *S. cerevisiae* can easily grow at 37°C (Salvadó *et al.*, 2011). *S. cerevisiae* is acidophilic so it grows better in acidic environments. The optimal pH range for fermentation of yeasts lies between pH 4 - 6 but they can even grow at a pH as low as 1.5. This all depends on the temperature, oxygen availability and type of strain that is used (Narendranath & Power, 2005).

Since the mean pH of a healthy human vagina lies around pH 4 it is no problem for *S. cerevisiae* to grow in this niche (O'Hanlon *et al.*, 2019). A yeast cell needs an energy source, like carbohydrates, so that all biological processes in the cell run normally and the yeast can grow. The preffered carbon source of *S. cerevisiae* is glucose. When this becomes depleted it can easily use a wide variety of alternatives such as galactose, maltose, melbiose, sucrose and also carbons that are not sugars like ethanol, glycerol, lactate, oleate and acetate. Before they can be used as energy source, they are first broken down to smaller molecules to eventually produce pyruvate during glycolysis. In this process ATP and NADH are produced without the use of oxygen. Yeasts can process pyruvate by both fermentation and respiration depending on the conditions. Under aerobic conditions, oxygen is used as final electron acceptor in respiration (Compagno *et al.*, 2014). Most organisms only use fermentation when oxygen is not available because respiration is energetically more favorable. *S. cerevisiae* is special in this aspect, it always prefers fermentation over respiration even when oxygen is available. So, *S. cerevisiae* will also ferment sugars in aerobic environments. *S. cerevisiae* uses a process that is energetically less favorable to outcompete other microorganisms. It naturally lives on fruits where there is a lot of microbial competition for fruit sugars. *S. cerevisiae* evolved the ability to rapidly converse sugars to ethanol when oxygen is present, so that other organisms can no longer use them and die from high ethanol levels. The yeast itself has a high ethanol tolerance (Dashko *et al.*, 2014). During fermentation not only ethanol is produced but also SCFA acetate and succinate. In 1.4.2.3, this production process is explained in more detail. After most sugars are depleted and *S. cerevisiae* becomes dominant, it can use the self-produced ethanol as carbon source during respiration. This metabolic shift from fermentation of glucose to respiration of ethanol is called the diauxic shift (Compagno *et al.*, 2014). As described in 1.2.1.4, there are various carbon sources present in the vagina that *S. cerevisiae* can use such as glucose, glycerol, lactate and acetate. The vagina is not completely anaerobic. There are areas that contain small concentrations of oxygen so it is generally considered a microaerobic environment (Medina-Colorado *et al.*, 2017). The organisms that live there need to be able to grow without oxygen but also need to be oxygen tolerant. These are ideal conditions for *S. cerevisiae* since it prefers anaerobic fermentation. These conditions also ensure that the main end products will be ethanol and SCFA. Both SCFA and ethanol have been demonstrated to inhibit the growth of *Candida* (Cottier *et al.*, 2015; Guinan *et al.*, 2019; Rane *et al.*, 2012). The effects of SCFA on *Candida* are explained in detail in 1.4.2.4. Besides carbon, nitrogen sources are also important for the growth of yeasts. They are needed for the production of amino acids, polyamines, nucleotide bases and derivatives. *S. cerevisiae* can use different nitrogen sources such as ammonium, amino acids, dipeptides and tripeptides. The preferred nitrogen-containing compounds of *S. cerevisiae* that support a high growth rate are ammonium, glutamate, asparagine and glutamine. Growth on proline, urea and allantoin on the other hand is rather slow (Crépin *et al.*, 2012).

There are different nitrogen sources present in the vagina that *S. cerevisiae* can use such as glutamate, glutamine, urea and proline (Amabebe *et al.*, 2016; Owen & Katz, 1999; Vicente-Muñoz *et al.*, 2020).

1.4.2.2. Anti-*Candida* effect of *S. cerevisiae*

S. cerevisiae strain CNCM I-3856 reduced the *C. albicans* vaginal load in women with VVC and increased the clearance of *C. albicans* from the vagina in mice (Cayzeele-Decherf *et al.*, 2017; Pericolini *et al.*, 2017). *In vitro*, this strain inhibited *C. albicans* adhesion to vaginal epithelial cells, induced *C. albicans* co-aggregation, inhibited *C. albicans* germ-tube and hyphae formation and reduced vaginal epithelial cell damage (Pericolini *et al.*, 2017). Strain CNCM I-3856 was also shown to suppress the expression of *SAP2* and *SAP6* genes in *C. albicans* both *in vitro* and in mice (Pericolini *et al.*, 2017). Saps are important virulence factors of *C. albicans* as they play a major role during adhesion to and invasion of the host cells (Naglik *et al.*, 2003). The suppression of Sap2 and Sap6 is specifically relevant for treatment of VVC due to their proinflammatory nature since vaginal inflammation is crucial in the pathogenesis of vaginal *Candida* infections (Pericolini *et al.*, 2017; Vecchiarelli *et al.*, 2015). Strain CNCM I-3856 also showed other therapeutic effects like reducing interleukin-8 production (IL-8), reducing the number of vaginal polymorphonuclear cells (PMNs) and enhancing the antimicrobial activity of PMNs (Gabrielli *et al.*, 2018). IL-8 is a key cytokine during inflammatory processes. It recruits PMNs, which release proinflammatory substances by degranulation (Gabrielli *et al.*, 2018; Lacy, 2006). These cells also have the capacity to produce diverse antimicrobial proteins and enzymes to kill small engulfed microorganisms, and release reactive oxygen species and cytokines to kill microorganisms extracellularly (Lacy, 2006). By reducing IL-8 and the number of PMNs in the vagina, *S. cerevisiae* can dampen local inflammation while maintaining or even enhancing the antimicrobial activity of the PMNs (Gabrielli *et al.*, 2018). Current research on vaginal *S. cerevisiae*-based probiotics is not focused on the beneficial effects of SCFA. However, this could be very interesting since one of the mechanisms by which *S. cerevisiae* potentially exerts its probiotic effects in the gut, is the production of SCFA (Ratajczak *et al.*, 2019; Schneider *et al.*, 2005; Sen & Mansell, 2020). SCFA produced by the intestinal microbiota play an important role in the modulation of inflammatory and immune responses not only in the gut but also in the rest of the body, by communicating with the immune system (Ratajczak *et al.*, 2019). SCFA can bind to free fatty acid receptors (FFAR) like for example FFAR2, FFAR3, hydroxycarboxylic acid receptor 2 and olfactory receptor 78, which are all G-protein coupled receptors (Ang *et al.*, 2018; Bolognini *et al.*, 2016; Ohira *et al.*, 2017; Pluznick *et al.*, 2013).

These FFAR can be found on epithelial cells of the gut, adipocytes, spleen cells, bone marrow cells, peripheral nervous system cells, kidney cells and different cells of the immune system cells such as macrophages, monocytes, dendritic cells and neutrophils (Brown *et al.*, 2003; D'Souza *et al.*, 2017; Kobayashi *et al.*, 2017; Le Poul *et al.*, 2003). Studies with mice demonstrated that the genetic deletion of FFAR can result in the development of diabetes, obesity, colitis, colon cancer, arthritis, hypertension and asthma (Ang *et al.*, 2018). Besides working via FFAR, SCFA are able to inhibit histone deacetylase enzymes (Chen *et al.*, 2003). This contributes to their immunomodulatory properties because histone deacetylases regulate the expression of genes that are known to promote the pathogenesis of many diseases such as cancer (Chen *et al.*, 2003; Xu *et al.*, 2007). It is also demonstrated in several studies that acetate, butyrate and propionate have an antifungal effect on *Candida* species (Lourenco *et al.*, 2018; Nguyen *et al.*, 2011; Yun & Lee, 2016). Focusing on high SCFA production together with other mechanisms-of-action during the development of vaginal *S. cerevisiae*-based probiotics could be advantageous.

1.4.2.3. Fatty acid production as probiotic mode of action

S. cerevisiae is capable of both producing and stimulating the production of SCFA and intermediates of fatty acid metabolism in other microbiota (Franco-Duarte *et al.*, 2017; Moens *et al.*, 2019; Offei *et al.*, 2019; Schneider *et al.*, 2005). SCFA are fatty acids with less than six carbon atoms, such as acetate (C_2), propionate (C_3) and butyrate (C_4). Examples of intermediates of fatty acid metabolism are succinate (C_4) and lactate (C_3). Depending on the pH, these fatty acids exist in undissociated form or as anion. As a consensus, and because the pKa values of these acids are close to the vaginal pH, they are always named in their anion form throughout this thesis. *S. cerevisiae* itself can produce acetate and succinate (Franco-Duarte *et al.*, 2017; Offei *et al.*, 2019). The production of both acids starts with the formation of pyruvate from a carbon source, for example glucose, via glycolysis (Figure 3). Acetate is formed during the fermentation of glucose to carbon dioxide. Pyruvate is first converted to acetaldehyde by pyruvate dehydrogenase. Acetate is then formed from acetaldehyde by acetaldehyde dehydrogenase. Acetaldehyde can also be converted to ethanol by alcohol dehydrogenase (Krivoruchko *et al.*, 2014). Succinate can be formed from pyruvate via three different pathways: the oxidative tricarboxylic acid cycle, the reductive branch of the tricarboxylic acid cycle and the oxidative pathway of the glyoxylate cycle (Raab & Lang, 2011). Franco-Duarte *et al.* (2017) found that there is an inverse correlation between the production of acetate and succinate in *S. cerevisiae*. Strains that produced the most succinate, had the lowest acetate production. This correlation could be important if both metabolites turn out to have an anti-*Candida* effect.

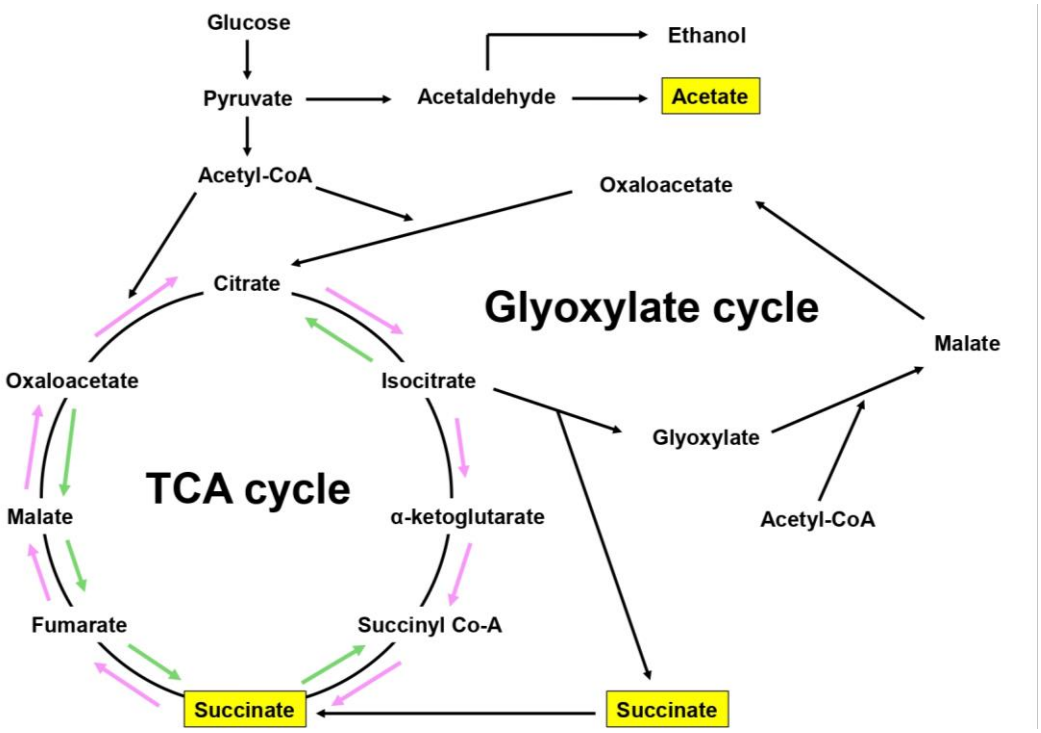


Figure 3: Schematic representation of acetate and succinate production metabolic pathways in *S. cerevisiae*. Pink arrows indicate the oxidative tricarboxylic acid (TCA) cycle. Green arrows indicate the reductive branch of the tricarboxylic acid (TCA) cycle. Based on (Franco-Duarte *et al.*, 2017; Krivoruchko *et al.*, 2014; Raab & Lang, 2011).

Besides its own production of fatty acid metabolites, *S. cerevisiae* can also stimulate the production of propionate and butyrate in other microorganisms. A study by Schneider *et al.* (2005) reported that a treatment with probiotic *Saccharomyces cerevisiae* var. *boulardii* increased fecal SCFA levels, especially butyrate, in patients on long-term total enteral nutrition which resulted in less diarrhea. Moens *et al.* (2019) showed in an *in vitro* colonic model system that *S. boulardii* stimulates the production of propionate in other microbiota. Because the concentration of *S. boulardii* decreased significantly 48 hours after addition to the colonic microbiota, it is thought that bacteria utilized cell wall components from lysed yeast cells as substrates to produce propionate. The results of Cuskin *et al.* (2015) and Hughes *et al.* (2008) confirm that propionate-producing bacteria can use major cell wall components of yeast like mannan-oligosaccharides and beta-glucans as substrates and that these components specifically stimulate the production of propionate. Pericolini *et al.* (2017) proved that dead *S. cerevisiae* cells can accelerate the clearance of *Candida* from the vagina in mice by forming a barrier on the vaginal epithelium and thereby preventing adhesion, and inducing *Candida* coaggregation. Thus, while living yeast cells can produce acetate and succinate, dead yeast cells can stimulate the production of SCFA in other microbiota, prevent adhesion and induce coaggregation.

1.4.2.4. Effect of fatty acids on *Candida*

Candida species can alter their central carbon metabolism to utilize lesser-preferred carbon sources like SCFA instead of glucose. This metabolic versatility is important for their virulence in niches like the vaginal tract that are often low in or deprived of glucose (Childers *et al.*, 2016). The entry of lactate (under the form of lactic acid, protonated) and SCFA in the fungal cells is expected to happen mainly via passive diffusion. The vaginal pH is close to or below the pKa of the weak acids ($pKa_{acetate}$ = 4.76; $pKa_{lactate}$ = 3.86; $pKa_{butyrate}$ = 4.82; $pKa_{propionate}$ = 4.88; $pKa_{succinate}$ = 4.21) so that they mostly occur in undissociated form (Lourenco *et al.*, 2018). Although in *C. albicans* and *C. glabrata* transporters are identified that mediate the uptake of lactate and/or acetate, it is unlikely that they play a crucial role in the tolerance to these acids (Lourenco *et al.*, 2018; Mota *et al.*, 2015; Vieira *et al.*, 2009). In *C. albicans*, SCFA have been shown to inhibit growth, germ tube formation, hyphae formation, hyphae attachment and reduce the metabolic activity of fungal cells in a biofilm. Most of these effects are partially caused by inducing acidic external conditions due to the dissociative properties of SCFA in addition to currently unknown mechanisms (Guinan *et al.*, 2019). An important conclusion that can be drawn from various studies is that an acidic environment is needed for the antimicrobial activity of SCFA (Aldunate *et al.*, 2015; Guinan *et al.*, 2019; Lai *et al.*, 2009; Lourenco *et al.*, 2018). In neutral conditions they exist in anion form, but no longer exhibit inhibitory effects. This conclusion is especially relevant in the vaginal niche, which naturally has an acidic pH. As previously stated, the inhibition of *Candida* growth by lactate is controversial. In various studies, the inhibitory effect of lactate on growth appears to be minor (Kasper *et al.*, 2015; Lourenco *et al.*, 2018; Moosa *et al.*, 2004), while in others, a clear effect is reported (Kohler *et al.*, 2012; Krasner *et al.*, 1956). The mechanisms by which SCFA exert their antifungal effects are not completely known yet. Various theories implicate intracellular acidification, accumulation of anions, ATP depletion and perturbation of the plasma membrane (Bracey *et al.*, 1998; Guldfeldt & Arneborg, 1998; Mollapour *et al.*, 2008; Stratford & Anslow, 1998; Ullah *et al.*, 2012). An overview of these effects is shown in Figure 4. To what extent each mechanism contributes to combined antifungal effects of various weak acids is not clarified yet. In model organism *S. cerevisiae*, the lipophilicity of weak acids correlates with the acidification rate of the cytosol, which confirms that weak acids need to diffuse over the plasma membrane to be toxic, and that not the initial acidification but rather the ability of the fungal cell to restore the intracellular pH is an important determinant for growth inhibition (Rane *et al.*, 2019). The longer the chain length of a weak acid, the higher the lipophilicity and its toxicity. The mechanism to restore the cytosolic pH seems specific for each weak acid. For acetate, the activity of Pma1p, a plasma membrane H^+-ATPase, is crucial for its resistance and long-term acidification is the major mechanism by which this weak acid inhibits growth. Although Pma1p activity is increased during acetate-stress, the capacity of the H^+-ATPase as well as the ATP availability are probably not limiting.

Acetate is as anion not very toxic. Its effect on the membrane integrity of *S. cerevisiae* is not yet fully clear. In Ullah *et al.* (2012), acetate does not significantly affect membrane integrity while Mira *et al.* (2010) reports that weak acids like acetate and lactate can cause membrane perturbation. *Candida* species have a better external pH adaptability than *S. cerevisiae* and can therefore survive in environments between pH 2-10 (Sherrington *et al.*, 2017). The cytosolic pH range is also different between *S. cerevisiae* and *Candida* species. In *S. cerevisiae* this range is between 6.0 and 7.0 while in *C. albicans* the intracellular pH range in vivo is wider, ranging between 5.8 and 9 (Kaur *et al.*, 1988; Rane *et al.*, 2019; Stewart *et al.*, 1989). So, intracellular acidification is probably not the only mechanism. Which other mechanisms are contributing to the inhibiting effect of fatty acid metabolites and to which extent, still needs to be elucidated.

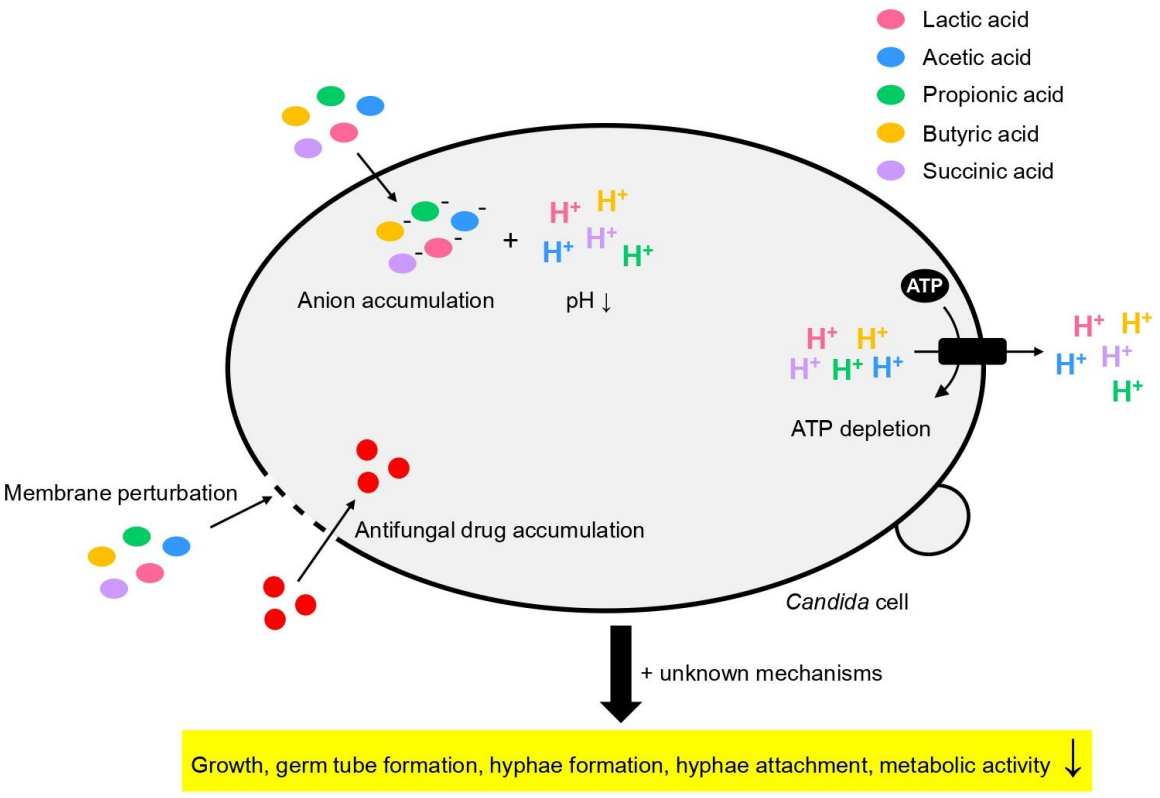


Figure 4: Schematic overview of effects of fatty acids on *Candida*.
Further explanation of the mechanisms is given in the main text. Adenosine triphosphate (ATP); Hydrogen ion (H^+). Based on (Bracey *et al.*, 1998; Guinan *et al.*, 2019; Guldfeldt & Arneborg, 1998; Mollapour *et al.*, 2008; Stratford & Anslow, 1998; Ullah *et al.*, 2012).

2. OBJECTIVES

This study is part of a larger project, ScerViCs (for *S. cerevisiae*-based treatment of vaginal candidiasis), which aims to develop a *S. cerevisiae*-based probiotic treatment of vulvovaginal *Candida* infections to reduce the general use of antifungal drugs and thus also the development of drug-resistant *Candida* species in patients. In contrast to other current research on vaginal *S. cerevisiae*-based probiotics, this study is focused on the potentially beneficial effects of *S. cerevisiae*-associated metabolites as probiotic mode-of-action. This study has three main objectives.

The first major objective is to investigate the effect of SCFA, directly or indirectly produced by *S. cerevisiae* (acetate, propionate and butyrate), on different *Candida* species in the vagina. This is investigated by performing growth assays of *Candida* in VSM supplemented with different concentrations of the different acids. A sub-objective is to compare the effect of *S. cerevisiae*-associated SCFA with the effect of *Lactobacillus*-associated lactate on *Candida* in the vagina. This is investigated by performing growth assays of *Candida* in VSM supplemented with different concentrations of lactate. To elicit the mode-of-action of lactate, its effect on *Candida* can be tested in VSM with varying acidity to find out whether its effect is pH-dependent. A comparison between the effects of L- and D-lactate on *Candida* can potentially help to understand why certain *Lactobacillus* species have a stronger antifungal effect compared to others. To investigate this, the same experimental setup can be used to perform growth assays.

The second objective is to investigate the combination effect of fluconazole, the most used antifungal drug in the treatment of vaginal fungal infections, and acetate on *Candida* in the vagina. First, the effect of fluconazole needs to be tested alone. This can be done by performing microdilution assays with *Candida* in VSM to determine the minimally inhibitory concentration. Once the azole susceptibility of the *Candida* species is verified in vaginal conditions, the combination of fluconazole and acetate can be tested. This can again be done with microdilution assays.

The third major objective is to validate the effectiveness of a *S. cerevisiae*-based probiotic treatment in an *in vivo* mouse model for vaginal *Candida* infections. For this objective, the mouse model for vaginal infections first needs to be optimized to obtain a stable vaginal *Candida* colonization. Using the optimized mouse model, a *S. cerevisiae*-based probiotic treatment can be compared to a fluconazole-based treatment of a vaginal *Candida* infection.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Media

3.1.1.1. 40% glucose

Glucose was used as carbon source in the YPD agar medium and the VSM medium. For this solution, 440 g glucose ($C_6H_{12}O_6.H_2O$) (Sigma-Aldrich) was dissolved in 1 L Milli-Q (Millipore Corporation) water, after which it was autoclaved at 120°C and 120 kPa for 20 minutes to sterilize the solution.

3.1.1.2. Yeast Extract Peptone Dextrose (YPD) agar medium

This medium was used to cultivate *C. albicans*, *C. glabrata* and *S. cerevisiae* strains. To make 1 L of YPD agar solution, 20 g/L bacteriological peptone (Thermo Scientific), 10 g/L granulated yeast extract (Merck) and 15 g/L granulated agar (Difco) was dissolved in 950 mL deionized water. To sterilize the solution, it was autoclaved at 120°C and 120 kPa for 20 minutes. Finally, 50 mL of 40% sterile glucose was added to the solution.

3.1.1.3. Vaginal simulative medium (VSM)

This medium (Owen & Katz, 1999) was used to cultivate *C. albicans*, *C. glabrata* and *S. cerevisiae* strains in conditions similar to the natural vaginal niche. For 1 L of standard VSM, 3.51 g/L NaCl (Sigma Aldrich), 1.40 g/L KOH (Sigma Aldrich), 0.222 g/L $Ca(OH)_2$ (Sigma Aldrich), 0.4 g/L urea (Sigma Aldrich), 1.7 or 0.2 g/L YNB (Difco) and 5 g/L ammonium sulphate (Honeywell) were dissolved in 900 mL Milli-Q water. In experiments 4.1.1.1, 4.1.1.2, 4.1.2.1, 4.1.3.1 and 4.1.3.2 the YNB concentration was 1.7 g/L. In experiments 4.1.1.3, 4.1.1.4, 4.1.1.5, 4.2.1 and 4.2.2 the YNB concentration was 0.2 g/L. After addition of 2 g/L D/L-lactic acid (85%) (Sigma Aldrich), 1 g/L acetic acid (100%) (Ensure) and 4 mL bovine serum albumin (BSA, prepared by dissolving 0.25 g BSA (Sigma Aldrich) in 50 mL Milli-Q), the pH was adjusted to 4.2 using HCl. Milli-Q water was added to get a total volume of 987,37 mL. Thereafter, the solution was sterilized by filtration with a 0.2 µm filter. Lastly, 12.5 mL/L of 40% sterile glucose (Sigma Aldrich) and 0.13 ml/L sterile glycerol (98%) (Sigma Aldrich) were added.

VSM with varying concentrations of acetic, lactic, propionic, butyric acid and varying pHs were also made. Besides these varying concentrations or pH, the composition was the same as the standard VSM. For experiments with acetic acid, VSM were made with concentrations of 0 g/L, 1 g/L, 2 g/L, 2.5 g/L, 3 g/L, 4 g/L and 5 g/L acetic acid (100%) (Ensure). For experiments with D/L-lactic acid, VSM were made with concentrations of 0 g/L, 2 g/L, 5 g/L, 10 g/L and 20 g/L D/L-lactic acid (85%) (Sigma Aldrich). For experiments with L-lactic acid, VSM were made with concentrations of 0 g/L, 2 g/L, 5 g/L, 10 g/L and 20 g/L L-lactic acid (≥ 98%) (Sigma Aldrich).

For experiments with D-lactic acid, VSM was made with a concentration of 5 g/L D-lactic acid (> 99%) (Bachem). For experiments with propionic acid, VSM were made with concentrations of 0 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L and 1 g/L propionic acid (≥ 99.5%) (Sigma Aldrich). For experiments with butyric acid, VSM were made with concentrations of 0 g/L, 0.2 g/L, 0.5 g/L, 1 g/L, 2 g/L and 5 g/L butyric acid (≥ 99.5%) (Sigma Aldrich). For experiments with varying pHs, the pH of the VSM were adjusted with HCl to 3, 3.5, 3.8, 4 and 4.2.

3.1.1.4. CHROMagar™

CHROMagar™ was used for the identification and counting of *Candida* and *S. cerevisiae* colonies. For 1 L of CHROMagar™, 47.7 g CHROMagar™ (CHROMagar) was dissolved in 1L Milli-Q. To sterilize, the solution was heated in an autoclave to a temperature of 110°C. After the temperature was reached, the autoclave was turned off to cool down.

3.1.2. Buffers

3.1.2.1. 1X Phosphate-buffered saline (PBS)

This buffer was used for washing *Candida* and *S. cerevisiae* cells. For 1 L of PBS, 8 g/L NaCl (Merck), 0.2 g/L KCl (VWR Chemicals), 1.44 g/L Na_2HPO_4 (Merck) and 0.24 g/L KH_2PO_4 (Merck) was dissolved in 1 L Milli-Q. The pH was adjusted to 7.4 using HCl. Finally, the solution was sterilized in an autoclave at 120°C and 120 kPa for 20 minutes.

3.1.3. Microbial strains

3.1.3.1. *Candida* species

Table 1: Overview of *Candida* strains used in the experiments.

Name	Species	Specification	Origin	Source	Biosafety level
Ca3153	*C. albicans*	Vagina?	Clinical isolate	(J. Yano & Fidel, 2011)	2
Ca41	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca42	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca43	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca44	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca45	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca46	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca47	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca48	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
Ca49	*C. albicans*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2
BG2	*C. glabrata*	Vagina	Clinical isolate	(Cormack & Falkow, 1999)	2
Cg28	*C. glabrata*	Vagina	Clinical isolate	Prof. Dr. Lagrou	2

3.1.3.2. *S. cerevisiae* strains

Table 2: Probiotic *S. cerevisiae* strain used in the experiments.

Name	Species	Specification	Origin	Biosafety level
6886	*S. cerevisiae*	Unknown	DBVPG	1

Abbreviations: DBVPG, Industrial Yeasts collection from the Department of Agricultural, Food and Environmental Science at the University of Perugia in Italy.

3.1.4. Mice strains

All experiments involving mice were approved by the ethical committee of KULeuven. BALB/cByJ mice have a higher reproductive performance, spontaneous dystrophic cardiac calcinosis, elevated free fatty acids, high triglyceride levels, and a number of hematologic abnormalities compared to BALB/cJ mice (Bogue & Grubb, 2004).

Table 3: Overview of mice strains used in the *in vivo* experiments.

Name	Species	Gender	Age	Origin
BALB/cJ	Mouse	Female	8 weeks	Janvier labs
BALB/cByJ	Mouse	Female	8 weeks	Charles River Laboratories

3.2. Methods

3.2.1. Precultures of microbial species

Cells of the different *Candida* and probiotic strains were obtained from the - 80°C stock, streaked on YPD Petri dishes and allowed to grow for 1 - 3 days in an incubator at 30°C. After this, precultures were made by picking up one colony and inoculating it in a test tube or Erlenmeyer flask with 3 – 50 ml standard VSM depending on how much cells were needed for the experiment. The test tubes and flasks were put in a shaking incubator at 240 rpm, at 30°C for one night.

3.2.2. *Candida* growth analysis – glass tubes

On the starting day of the experiment, the cells of the precultures were washed three times with sterile 1X PBS. After washing, the concentration of the cells was determined using a spectrophotometer (Eppendorf BioPhotometer). The optical density of the cells was measured at 600 nm (OD_{600}). The cells were then diluted to an OD_{600} value of 0.2 in new glass tubes with 4 mL of VSM. Depending on which experiment was done, these VSM contained different concentrations of lactic or acetic acid. These glass tubes were then put in shaking incubator at 240 rpm, at 37°C. After 8, 24 and 48 hours the OD_{600} was measured with a spectrophotometer (Eppendorf BioPhotometer).

3.2.3. *Candida* growth analysis – Multiskan™

On the start day of the experiment, the cells of the precultures were washed three times with sterile Milli-Q. After washing, the concentration of the cells was determined using a spectrophotometer (Eppendorf BioPhotometer) at OD_{600}. The cells were then diluted in Eppendorf tubes of 1.5 mL to an OD_{600} value of 0.2 with sterile Milli-Q. Next, the cells and media were put in a sterile 96-well plate. To every well, 10 µL of cells and 190 µL of VSM was added. Depending on which experiment was done, these VSM contained different concentrations of lactic, acetic, propionic or butyric acid. A sealing sticker was put on the 96-well plate, after which it was placed in a Multiskan™ (Thermo Fisher Scientific) for 75 hours at 37°C. Every half hour, the OD_{600} was measured by the Multiskan™.

3.2.4. Azole susceptibility – Minimum Inhibitory Concentration (MIC)

On the start day of the experiment, the cells of the precultures were washed three times with sterile 1X PBS. After washing, the concentration of the cells was determined using a spectrophotometer (Eppendorf BioPhotometer) at OD_{600}. The cells were then diluted with sterile 1X PBS in Eppendorf tubes of 1.5 mL to an OD_{600} value of 0.2 for *C. albicans* and 0.15 for *C. glabrata*. The cells were further diluted in 5 mL standard VSM or VSM with different concentrations of acetic acid in glass tubes by adding 10 µL of *C. albicans* cells or 7 µL of *C. glabrata* cells. As a control, 20 µl of each cell suspension was plated out on a YPD plate, which was then incubated at 37°C for 24 hours.

To prepare the fluconazole dilution series, Eppendorf tubes of 1.5 ml with 2.5 mg/mL fluconazole were thawed from the – 20°C stock. For *C. albicans*, dilution series were made in standard VSM or VSM with different concentrations of acetic acid to obtain concentrations of 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µg/mL fluconazole. For *C. glabrata*, dilution series were made in standard VSM or VSM with different concentrations of acetic acid to obtain concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 µg/mL fluconazole.

When the cell suspensions and fluconazole dilution series were made, everything was put in a sterile 96-well plate. First, 80 µL standard VSM or VSM with different concentrations of acetic acid was added to each well. Then, 20 µL of fluconazole was added to every well. Finally, 100 µL of the cell suspensions were added to each well. Every plate contained only one type of VSM and every column contained a different concentration of fluconazole. Every plate contained three biological and two technical repeats. Aluminium foil was wrapped around the 96-well plates, after which they were placed in an incubator at 37°C for 48 hours. After the incubation, the cells were resuspended with a pipet. Then, the OD_{600} was measured of the plates with Synergy™ H1 (BioTek), which is a hybrid multi-mode microplate reader.

3.2.5. Azole synergy with acetate – Checkerboard Assay

The preparations of the *Candida* cell suspensions, the fluconazole dilution series and the volumes in the 96-well plates were the same as in 3.2.4. Every row contained VSM with a different concentration of acetic acid and every column a different concentration of fluconazole. Every plate contained only one biological and one technical repeat. Aluminium foil was wrapped around the 96-well plates, after which they were placed in an incubator at 37°C for 48 hours. After the incubation, the cells were resuspended with a pipet. Then, the OD_{600} was measured of the plates with Synergy™ H1 (BioTek).

The fractional inhibitory concentration (FIC) index was used to determine the interaction of fluconazole and acetic acid. The MIC_{50} and MIC_{90} values were determined by looking at which fluconazole and/or acetic acid concentration the growth of *Candida* dropped below an OD_{600} value of respectively 0.5 and 0.1. The FIC index was calculated according to the following equation:

$$(MIC_{combination}/MIC_{fluconazole}) + (MIC_{combination}/MIC_{acetic\ acid}) = FIC_{fluconazole} + FIC_{acetic\ acid} = \text{FIC index}$$

3.2.6. *In vivo* validation of probiotic strains

All mice experiments were ethically approved by the KU Leuven commission for animal experimentation.

3.2.6.1. Estradiol injections

The mice were injected with estradiol to induce pseudo-estrus. A thicker vaginal wall assures a long, stable vaginal *Candida* infection. To prepare the estradiol, 25 mg β-estradiol 17-valerate (≥ 98%) (Sigma Aldrich) was dissolved in 1 mL sesame oil in a 1.5 mL Eppendorf tube. The solution was mixed well, after which it was put in a shaking incubator at 37°C on 500 rpm for 15 minutes. Then, it was put in a sonicator (Branson 2210) for 10 minutes. After the sonication, sesame oil was added to a total volume of 5 mL. Lastly, the solution was heated to 37°C and mixed.

For the injections, the mice were first sedated using isoflurane. Each mouse was injected subcutaneously in the lower abdomen with 100 µL using a 16 mm needle. After about 4 minutes the needle was slowly removed from the mouse. Finally, the isoflurane sedation was stopped and the mouse was left to recover.

3.2.6.2. *Candida* infection and probiotic treatment

The cells of the *Candida* and *S.* cerevisiae precultures were washed three times with sterile 1X PBS. After washing, the concentration of the cells was determined using a spectrophotometer (Eppendorf BioPhotometer) at OD_{600}. The cells were then diluted with sterile 1X PBS to an OD_{600} value of 0.05. From this dilution, 200 µL was added to a 96-well plate. The 96-well plate was put in a Guava® easyCyte™ Flow Cytometer (Luminex) to accurately determine the cell concentration. The cells were then diluted with sterile 1X PBS to the desired concentration.

For the infection, the mice were first sedated by injecting 75 mg/kg ketamine and 1 mg/kg medetomidine. Depending on the experiment, the mice were infected using a cotton swab or a pipet. Cotton swabs were submerged in a *Candida* cell suspension of $2.5x10^6$ cells/mL and soaked for 2 minutes. The swabs were placed in the vaginas of the sedated mice for 30 minutes. This was repeated one more time. In the second setup, 10 µL of the *Candida* cell suspension ($2.5x10^6$, 10^7 or 10^8 cells/mL) was pipetted in the vaginas of the sedated mice. The mice were laying on their back for 1 hour. After the infection, the mice were injected with atipamezole (Antisedan) to recover.

For the treatment, fluconazole (≥ 98%) (Sigma Aldrich) was added to sterile 1X PBS to a concentration of 200 µg/mL. The mice were first sedated by injecting 75 mg/kg ketamine and 1 mg/kg medetomidine. With a pipet, 10 µL of the *S. cerevisiae* cell suspension or fluconazole solution was put in the vaginas of the sedated mice. The mice were laying on their back for 1 hour. After the infection, the mice were injected with atipamezole (Antisedan) to recover.

3.2.6.3. Vaginal lavages

During the vaginal lavage of a mouse, the vagina was washed twice with 50 µL 1X PBS. The PBS with vaginal fluid was put in a 1.5 mL Eppendorf tube. Dilution series were made in sterile 1X PBS to 1, 1:10, 1:100 and 1:1000. These dilutions were then plated out on CHROMagar™ plates. The plates were placed in an incubator for 2 to 3 days at 37°C. After the incubation, the colony forming units (CFU) were counted.

4. RESULTS

4.1. *In vitro* experiments

4.1.1. Effect of fatty acid metabolites on the growth of *Candida*

4.1.1.1. Lactate and acetate differentially affect growth of *Candida*

C. albicans strain Ca3153 and *C. glabrata* strain BG2 were grown in glass tubes (under continuously shaking conditions) that contained VSM with different concentrations of lactate or acetate. At 4, 6, 8, 24 and 48 hours the OD_{600} was measured with a spectrophotometer. The results of these growth experiments can be seen in Figure 5.

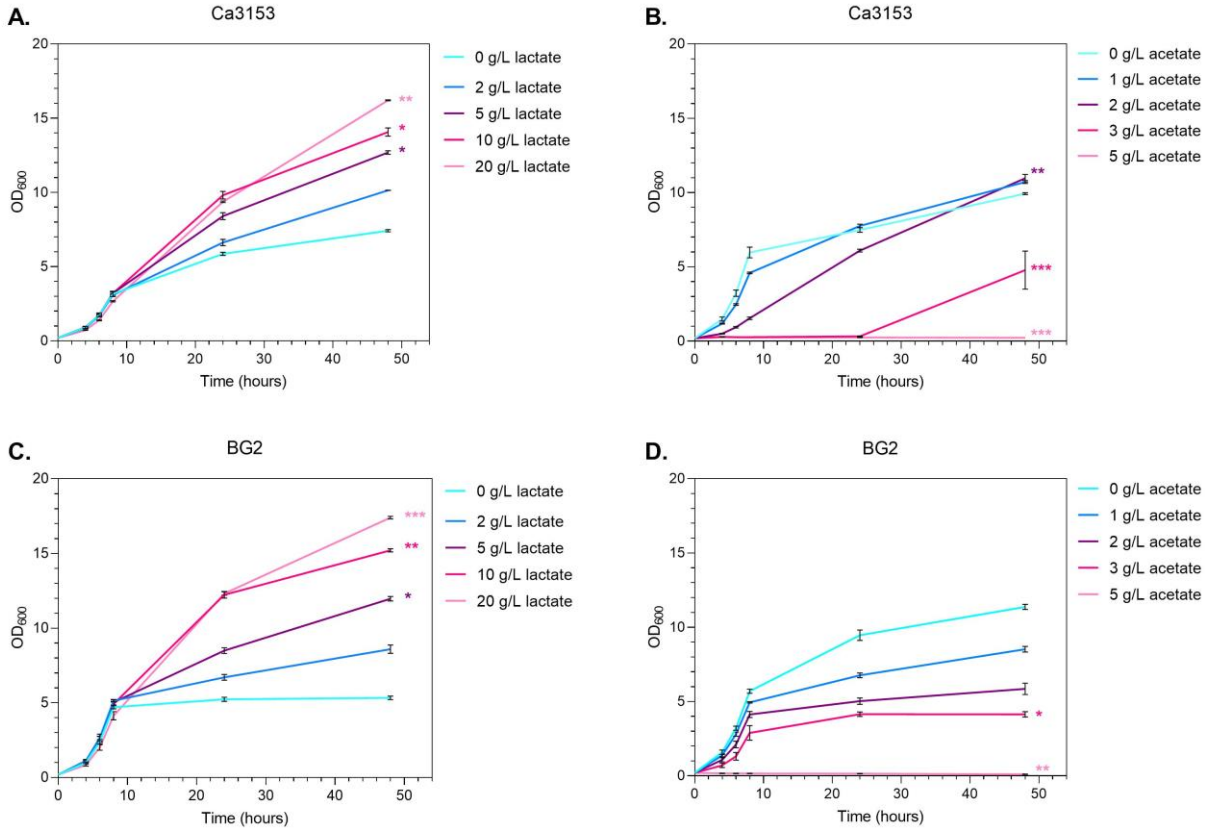


Figure 5: Growth curves of *C. albicans* strain Ca3153 (A, B) and *C. glabrata* strain BG2 (C, D) in VSM with different concentrations of lactate (left) or acetate (right) performed in glass tubes. Data are expressed as the average of three biological repeats ± SEM. The statistical significance was tested with Paired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a treatment group and the control (0 g/L) at 24 hours.
$P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

In Figure 5A and 5B, the growth curves of Ca3153 are shown. At 24 hours, the OD_{600} of Ca3153 in VSM containing 5, 10 and 20 g/L lactate is significantly higher compared to 0 g/L lactate. A stimulating trend of lactate on *C. albicans* can clearly be seen. The opposite can be seen in Figure 5B. At 24 hours, the OD_{600} of Ca3153 in 2, 3 and 5 g/L acetate is significantly lower compared to 0 g/L acetate. An inhibiting trend of acetate on *C. glabrata* is clear. In Figure 5C and 5D, the growth curves of BG2 are shown. At 24 hours, the OD_{600} of BG2 in 5, 10 and 20 g/L lactate is significantly higher compared to 0 g/L lactate. So, a stimulating trend of lactate can also be seen on *C. glabrata*. Again, the opposite can be seen in Figure 5D. At 24 hours, the OD_{600} of BG2 in 3 and 5 g/L acetate is significantly lower compared to 0 g/L acetate. An inhibiting trend of acetate on *C. glabrata* is also clear.

To make the experiments more high-throughput, the same experiments were also performed in 96-well plates with a Multiskan™. Other reasons for the use of a Multiskan™ is the possibility of including more biological repeats and more frequent measurements while making the experiments less labor intensive. It is noteworthy to mention that the strains were grown under continuous shaking conditions and a sticker was applied to the plates to avoid evaporation. *C. albicans* strain Ca3153 and *C. glabrata* strain BG2 were grown in a 96-well plate that contained VSM with different concentrations of lactate or acetate. For 75 hours, every half hour, the OD_{600} was measured by the Multiskan™. The results of these growth experiments can be seen in Figure 6.

In Figure 6A and 6C, the growth curves of Ca3153 and BG2 are shown in different concentrations of lactate. At 24 hours, there is no significant difference between the OD_{600} of Ca3153 and BG2 in any of the concentrations of lactate compared to 0 g/L lactate. Thus, in contrast to the glass tubes, no effect of lactate on either *C. albicans* or *C. glabrata* can be seen in the 96-well plate setup. In Figure 6B, the OD_{600} of Ca3153 in 1, 3 and 5 g/L acetate is significantly lower compared to 0 g/L acetate at 24 hours. So, just like in the glass tubes, an inhibiting trend of acetate can be seen on *C. albicans*. In Figure 6D, the OD_{600} of BG2 in 2, 3 and 5 g/L acetate is significantly lower compared to 0 g/L acetate at 24 hours. So, also in the 96-well plate setup an inhibiting trend of acetate can be seen on *C. glabrata*.

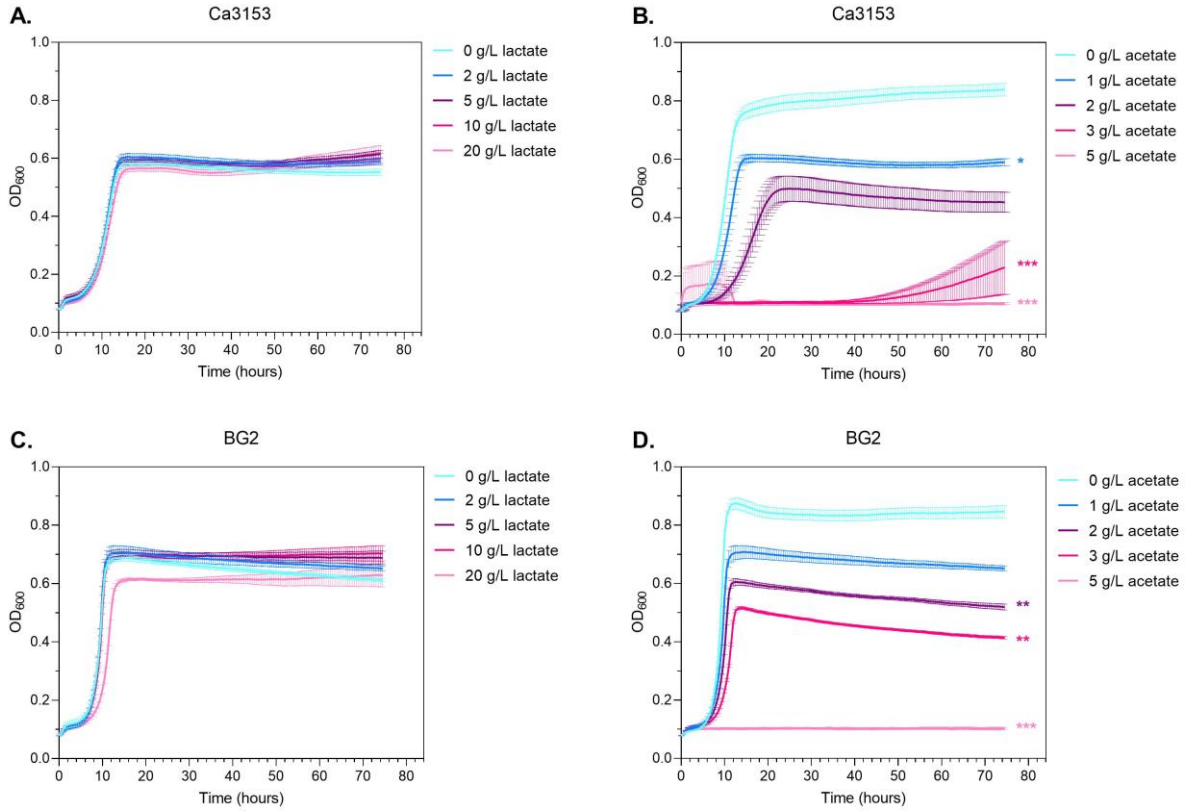
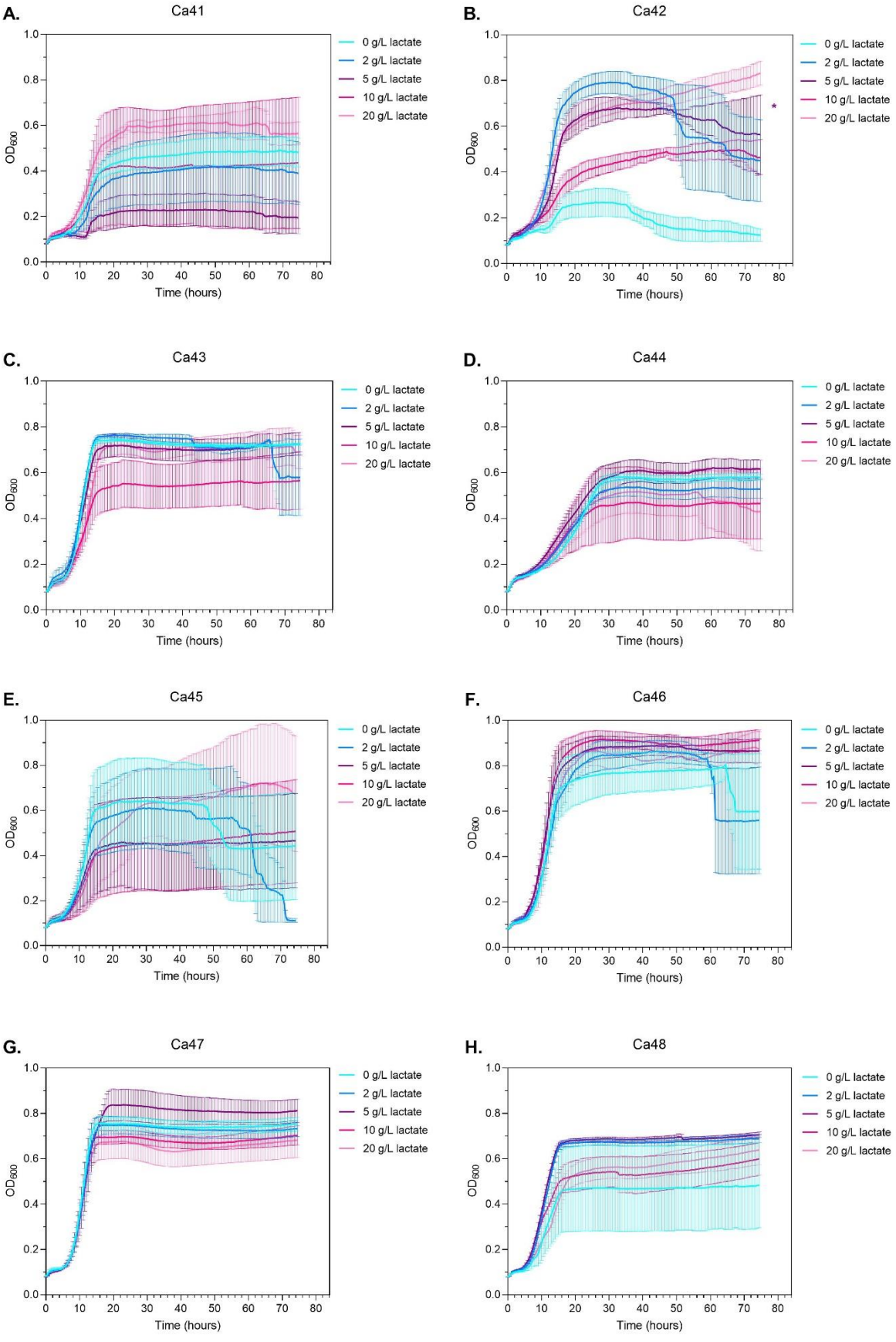


Figure 6: Growth curves of *C. albicans* strain Ca3153 (A, B) and *C. glabrata* strain BG2 (C, D) in VSM with different concentrations of lactate (left) or acetate (right) performed in Multiskan™. Data are expressed as the average of four biological repeats ± SEM. The statistical significance was tested with Paired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a treatment group and the control (0 g/L) at 24 hours.
$P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

4.1.1.2. Acetate but not lactate affects growth of clinical *Candida* isolates

The effect of lactate and acetate was also tested on other *Candida* strains to verify if the same trends could be seen. *C. albicans* strains Ca41, Ca42, Ca43, Ca44, Ca45, Ca46, Ca47, Ca48, Ca49 and *C. glabrata* strain Cg28 were grown in 96-well plates in VSM with different concentrations of lactate or acetate. For 75 hours, every half hour the OD_{600} was measured by the Multiskan™. The results of these growth experiments can be seen in Figure 7 and 8.

There are no statistically significant differences in Figure 7, except in Figure 7B. The OD_{600} of Ca42 in 5 g/L lactate is significantly higher than the control at 24 hours. There is quite some variation in the figures of certain strains.


A.
Ca41
B.
Ca42
C.
Ca43
D.
Ca44
E.
Ca45
F.
Ca46
G.
Ca47
H.
Ca48
OD600
Time (hours)
0 g/L lactate
2 g/L lactate
5 g/L lactate
10 g/L lactate
20 g/L lactate
*

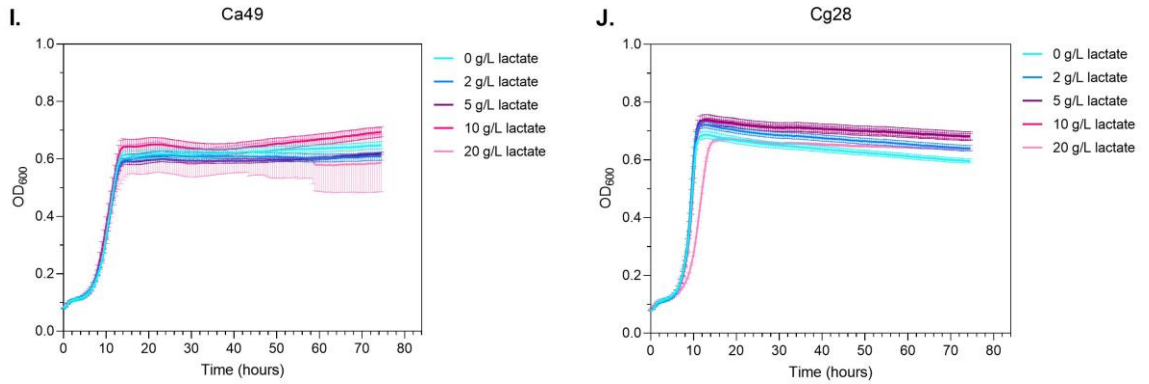
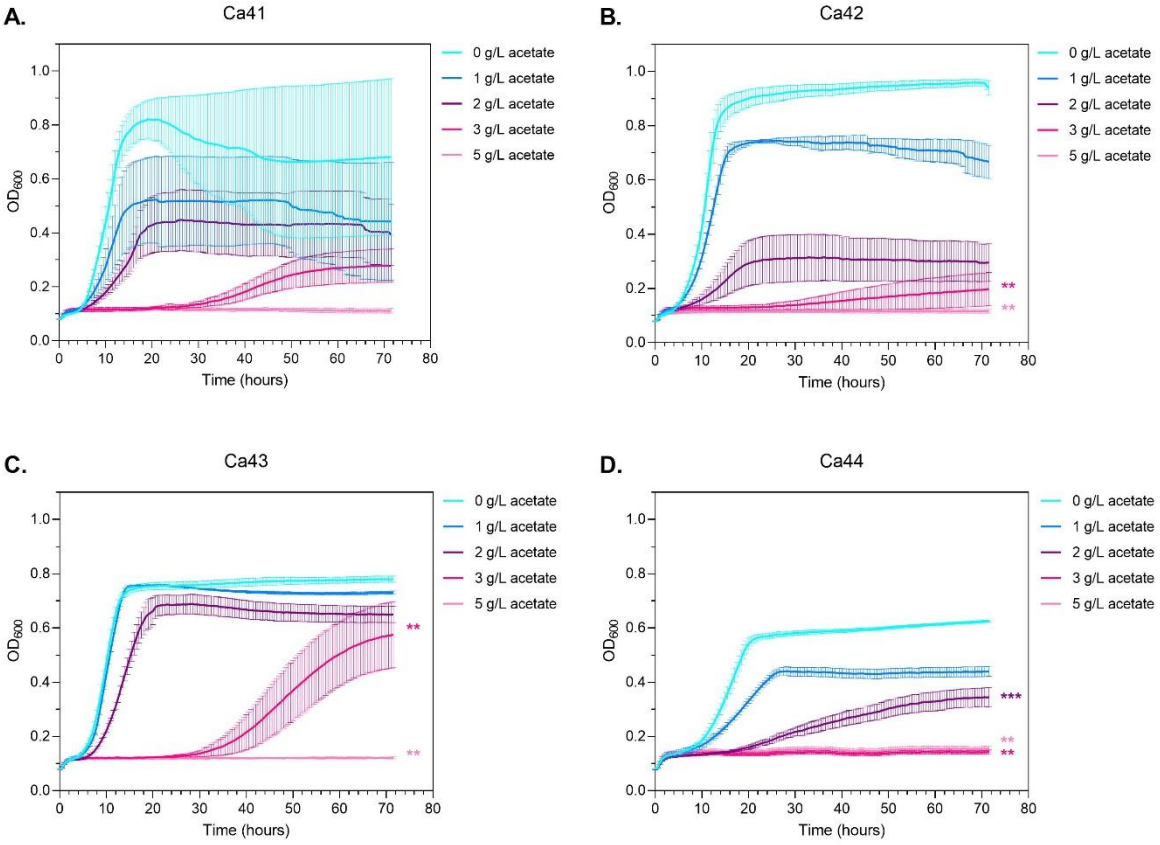


Figure 7: Growth curves of clinical *Candida* isolates in VSM with different concentrations of lactate performed in Multiskan™. Data are expressed as the average of three biological repeats ± SEM. The statistical significance was tested with Paired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a treatment group and the control (0 g/L) at 24 hours. P < 0.05 (*), P < 0.005 (**), P < 0.001 (***)

In Figure 8, the OD_{600} at 24 hours of Ca42, Ca43, Ca44, Ca45, Ca46 and Ca49 in 3 and 5 g/L acetate is significantly lower compared to the control. The OD_{600} at 24 hours of Ca44 in 2 g/L is also significantly lower compared to the control. The OD_{600} of *C. glabrata* strain Cg28 at 24 hours is significantly lower in 1, 2 and 5 g/L acetate compared to the control. Even for the strains where no statistically significant difference could be computed, an overall inhibiting trend of acetate can be seen on the growth of all *Candida* isolates that were tested.



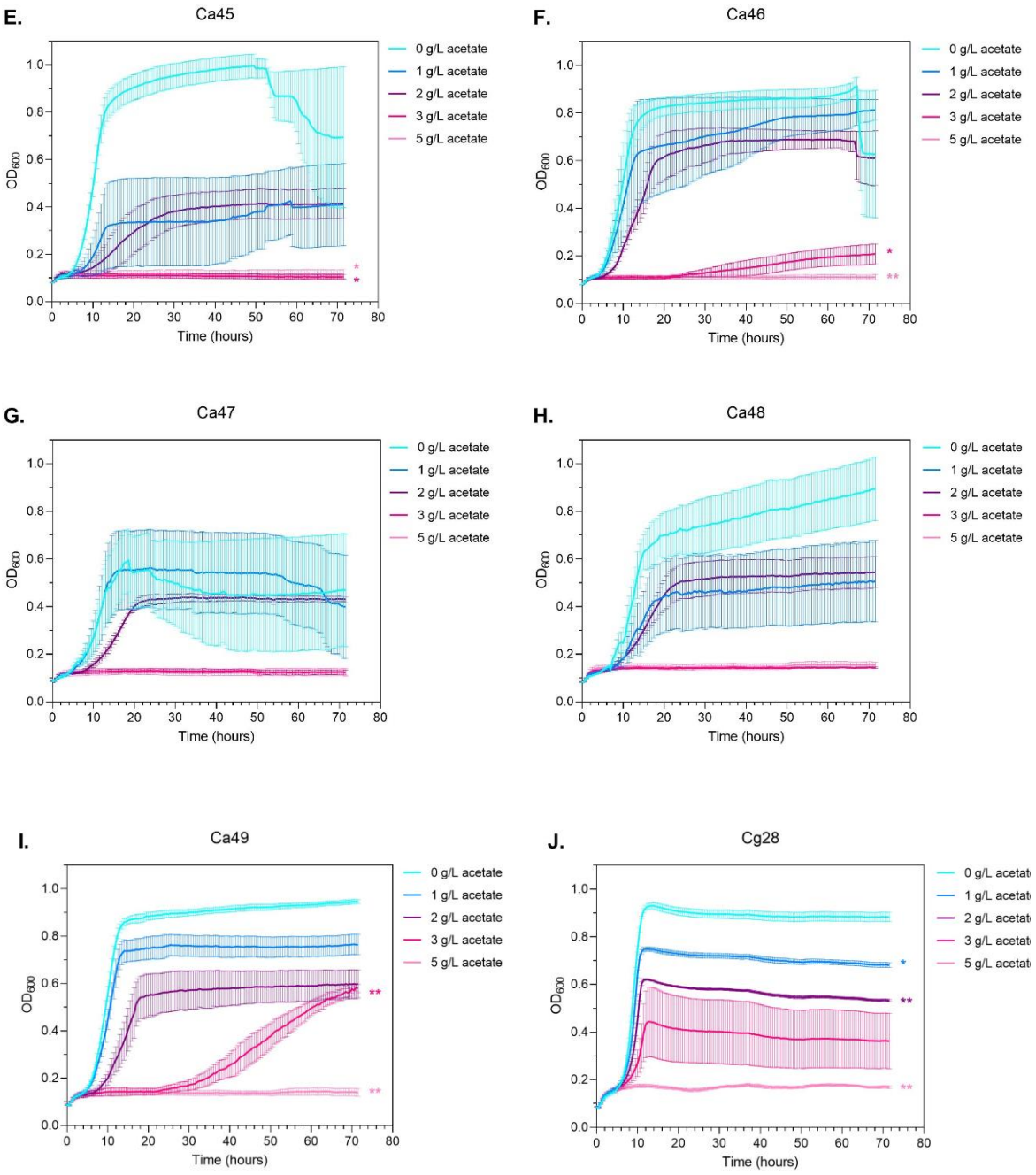


Figure 8: Growth curves of clinical *Candida* isolates in VSM with different concentrations of acetate performed in Multiskan™. Data are expressed as the average of three biological repeats ± SEM. The statistical significance was tested with Paired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a treatment group and the control (0 g/L) at 24 hours. $P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

4.1.1.3. Effect of lactate on *Candida* growth is not pH-dependent

To explain the lack of inhibitory effects of lactate in both the glass tubes and the 96-well plate setup, a hypothesis was tested. One hypothesis to explain the contradictory and non-consistent effects of lactate in different studies and experimental set-ups, including this study, is that the effect of lactate could be pH dependent (Kasper *et al.*, 2015; Kohler *et al.*, 2012). The pKa of lactate is 3.86, while the pH of the VSM that is used in previous experiments is 4.2. By lowering the pH of the VSM, more molecules of lactate will be undissociated and maybe only then have an inhibitory effect on the growth of *Candida*. In this experiment the pH of the VSM was lowered to 3, 3.5 and 3.8 for the glass tubes and to 3.5, 3.8, 4 and 4.2 for the Multiskan^{TM}. Because of technical difficulties, not all pHs were tested in the glass tubes. Half of the VSM contained 2 g/L lactate and the other half contained no lactate to make sure that the effects were not caused by the low pH alone. For 75 hours, every half hour the OD_{600} was measured by the Multiskan^{TM} and at 24 and 48 hours the OD_{600} from the glass tubes was measured with a spectrophotometer. The growth curves of *C. albicans* strain Ca3153 and *C. glabrata* strain BG2 in these media can be seen in Figure 9.

Although there are no significant differences in Figure 9A and C, a slight trend can be seen that the OD_{600} of Ca3153 and BG2 is higher in VSM with lactate compared to no lactate. In Figure 9B, the OD_{600} of Ca3153 is significantly higher at 24 hours in VSM with lactate compared to no lactate at pH 3.5 and 3.8. In Figure 9D, the OD_{600} of BG2 at 24 hours is significantly higher in VSM with lactate compared to no lactate at pH 3.5, 3.8 and 4. For Figure 9A, B, C and D, the significance of the differences between the groups that contained lactate was also tested with one-way ANOVA tests with Bonferroni correction but they were all non-significant.

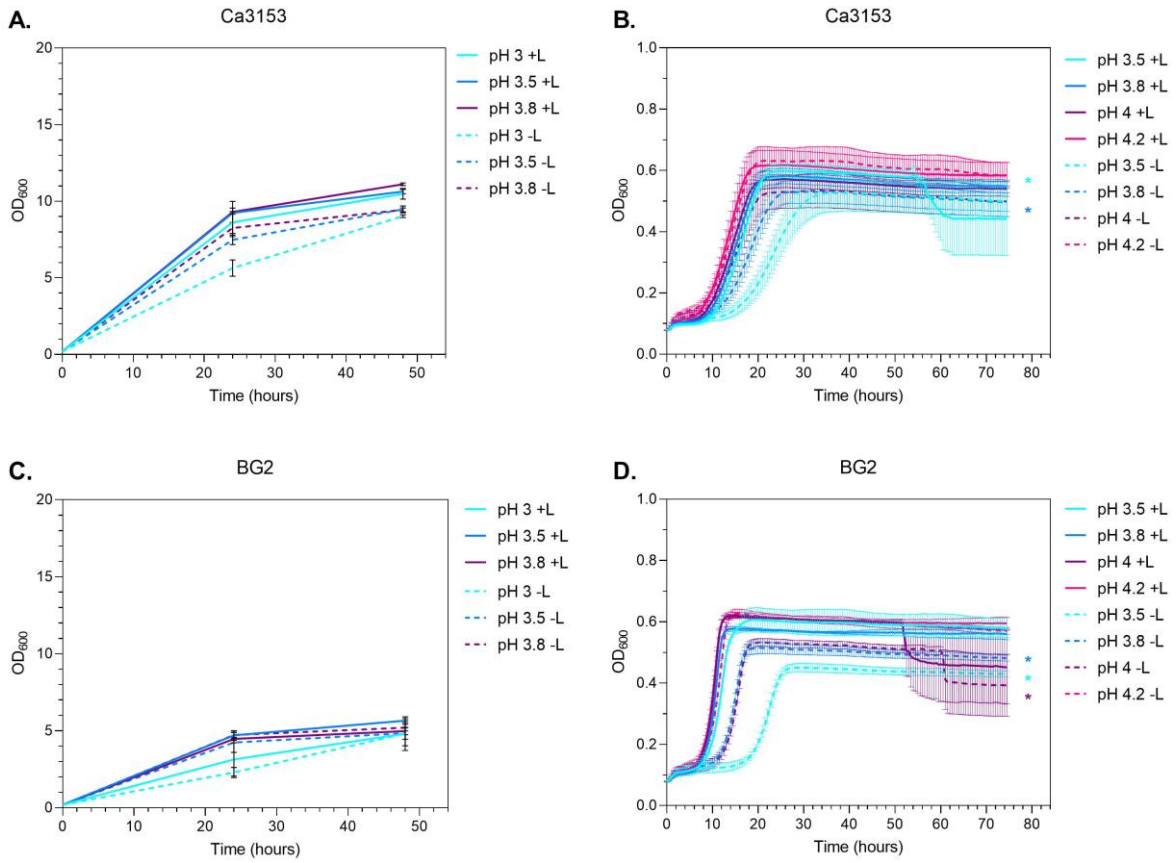


Figure 9: Growth curves of *C. albicans* strain Ca3153 (A, B) and *C. glabrata* strain BG2 (C, D) in VSM with varying pHs performed in glass tubes (left) and Multiskan™ (right). Solid lines represent *Candida* growth in VSM with 2 g/L lactate. Dotted lines represent *Candida* growth in VSM without lactate. Data of **(A)** and **(C)** are expressed as the average of three biological repeats ± SEM. Data of **(B)** and **(D)** are expressed as the average of four biological repeats ± SEM. The statistical significance was tested with Paired t-tests with Bonferroni correction. An asterisk indicates a significant difference between a group with and without lactate within the same pH at 24 hours.
$P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

4.1.1.4. No significant difference between the effects of L- and D-lactate on *Candida* growth

From literature it is known that not all lactate-producing bacteria are able to produce both the L- and D-isomer of lactate and that they not all have an equally inhibiting effect on *Candida* growth. That is why the difference between the effect of L- and D-lactate was tested. In all the other experiments of this study, a mixture of L- and D-lactate was used. *C. albicans* strain Ca3153 and *C. glabrata* strain BG2 were grown in VSM with different concentrations of L- or D-lactate. At 24 and 48 hours the culture OD_{600} from the glass tubes was measured with a spectrophotometer. Every half hour the culture OD_{600} was measured from the 96-well plates by the Multiskan™ for 75 hours. The results of these growth experiments can be seen in Figure 10.

For Ca3153 (Figure 10A and B), the OD_{600} at 24 hours in VSM with 20 g/L L-lactate in the 96-well plate setup is significantly higher compared to the control. For BG2 (Figure 10C), the OD_{600} at 24 hours is significantly higher in VSM with 20 g/L L-lactate and with 5 g/L D-lactate in the glass tubes. For Figure 10A, B, C and D, the significance of the difference between 5 g/L L-lactate and 5 g/L D-lactate was also tested with Paired t-tests with Bonferroni correction but they were all non-significant. Although there are not a lot of significant differences, there is a slight trend visible that especially D-lactate has a stimulating effect on the growth of *Candida*.

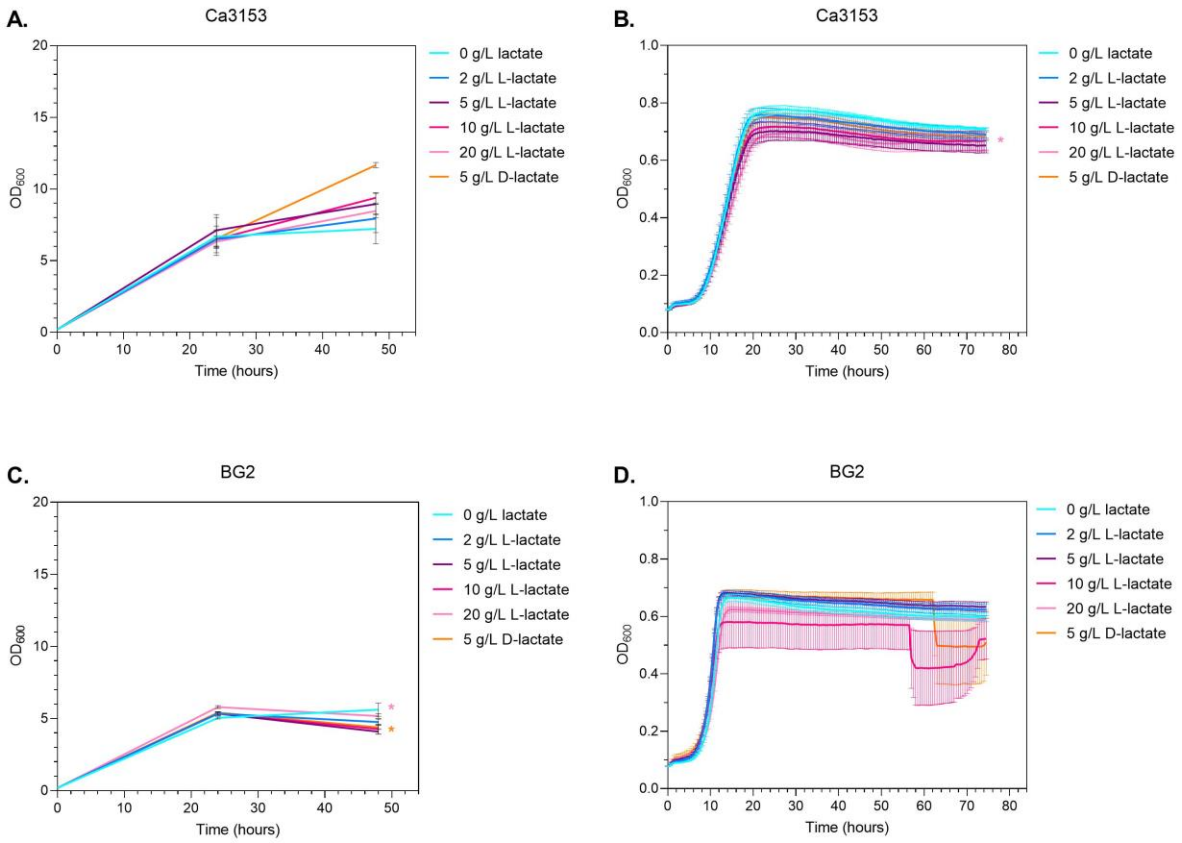


Figure 10: Growth curves of *C. albicans* strain Ca3153 (A, B) and *C. glabrata* strain BG2 (C, D) in VSM with L- or D-lactate performed in glass tubes (left) and Multiskan™ (right). Data of **(A)** and **(C)** are expressed as the average of three biological repeats ± SEM. Data of **(B)** and **(D)** are expressed as mean of four biological repeats ± SEM. The statistical significance was tested with Paired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a treatment group and the control (0 g/L) at 24 hours.
$P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

4.1.1.5. Propionate and butyrate inhibit *Candida* growth

From literature it is known that *S. cerevisiae* can stimulate the production of propionate and butyrate by other microorganisms. Here, *C. albicans* strain Ca3153 and *C. glabrata* strain BG2 were grown in a 96-well plate that contained VSM with different concentrations of propionate or butyrate to test their effect on *Candida* growth. For 75 hours, every half hour the OD_{600} was measured by the Multiskan™. The results of these growth experiments can be seen in Figure 11.

For Ca3153 (Figure 11A and B), the OD_{600} at 24 hours in VSM with 0.5 and 1 g/L propionate, and VSM with 1, 2 and 5 g/L butyrate are significantly lower compared to the control. For BG2 (Figure 11C and D), the OD_{600} at 24 hours in VSM with 0.2, 0.5 and 1 g/L propionate, and VSM with 0.2, 0.5, 1, 2 and 5 g/L butyrate are significantly lower compared to the control. In Figure 11 can clearly be seen that both propionate and butyrate effectively inhibit the growth of *C. albicans* and *C. glabrata*.

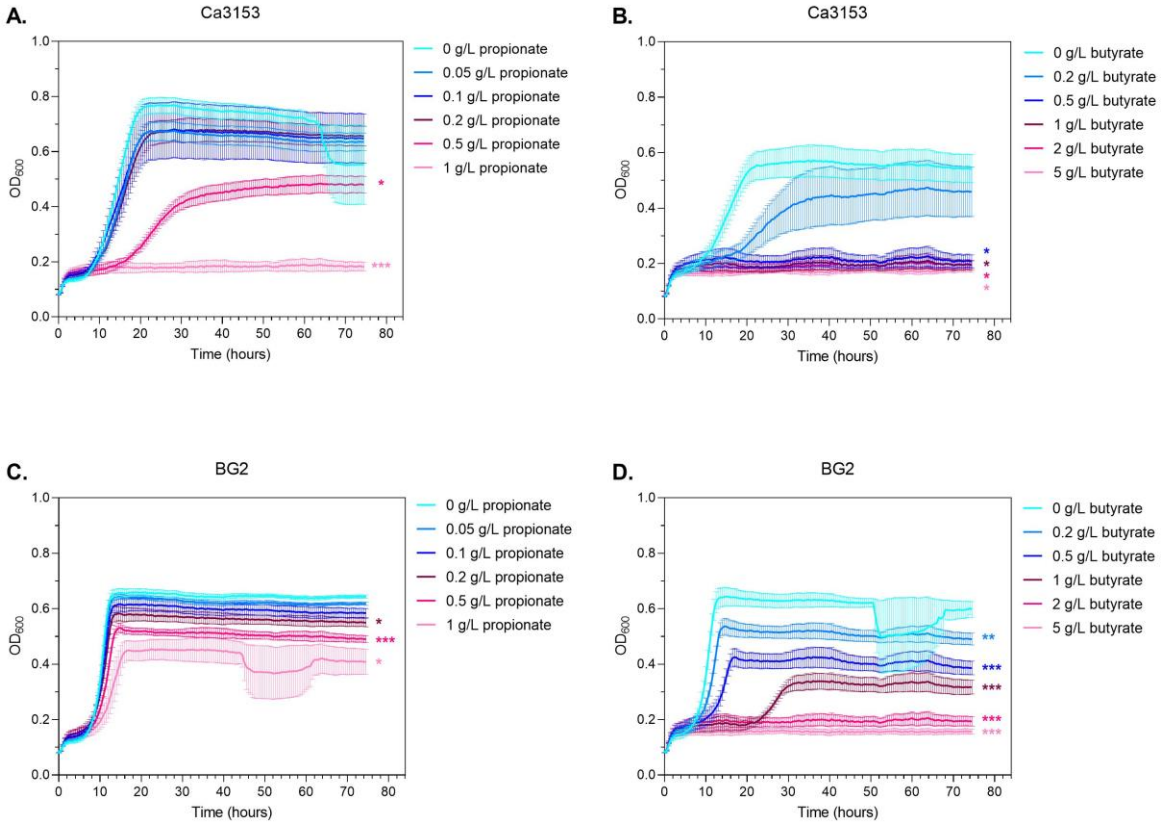


Figure 11: Growth curves of *C. albicans* strain Ca3153 (A, B) and *C. glabrata* strain BG2 (C, D) in VSM with propionate (left) or butyrate (right) performed in Multiskan™. Data are expressed as the average of four biological repeats ± SEM. The statistical significance was tested with Paired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a treatment group and the control (0 g/L) at 24 hours.
$P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

4.1.2. Azole susceptibility

4.1.2.1. BG2 is less susceptible to fluconazole compared to Ca3153 in VSM

With a view to testing the synergy between acetate and fluconazole, the susceptibility of *C. albicans* strain Ca3153 and *C. glabrata* strain BG2 was first tested to fluconazole alone. Minimum inhibitory concentration (MIC) tests were done with different concentrations of fluconazole in VSM. The results of these tests are shown in Figure 12. The MIC_{50} value represents the concentration of fluconazole at which the OD_{600} value of the growth culture drops below 0.5. The MIC_{90} value represents the concentration of fluconazole at which the OD_{600} value of the growth culture drops below 0.1. The MIC_{50} of Ca3153 and BG2 are respectively 1 µg/mL and 128 µg/mL fluconazole. The MIC_{90} of Ca3153 is also 1 µg/mL while that of BG2 is more than 128 µg/mL. From Figure 12 it is clear that *C. glabrata* is less susceptible to fluconazole compared to *C. albicans*.

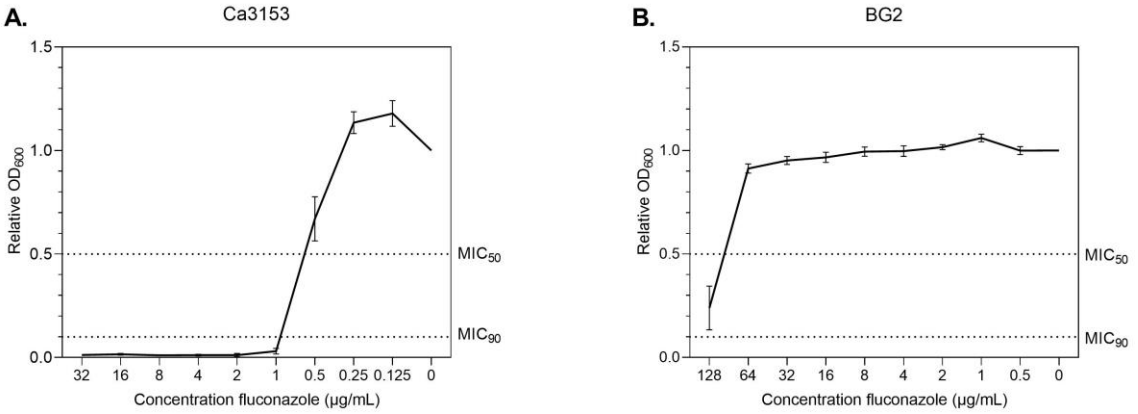


Figure 12: MIC curves of Ca3153 (A) and BG2 (B) in VSM with different concentrations of fluconazole. Data are expressed as mean of four biological repeats ± SEM. MIC_{50} and MIC_{90} are indicated by horizontal dotted lines.

4.1.3. Azole synergy with acetate

4.1.3.1. MIC test indicates potential synergism between fluconazole and acetate

The combined effect of fluconazole and acetate was tested on the growth of *C. albicans* strain Ca3153 and *C. glabrata* strain BG2 with MIC tests. The results of these experiments are shown in Figure 13. The MIC_{50} of Ca3153 in VSM without acetate is 0.25 µg/mL fluconazole, while in VSM with 1, 2 and 3 g/L the MIC_{50} of Ca3153 is 0.125 µg/mL. The MIC_{90} is more difficult to name due to technical variation. The MIC_{50} of BG2 in VSM with 0, 2 and 3 g/L acetate is 64 µg/mL fluconazole. In VSM with 1 g/L acetate, the MIC_{50} of BG2 is 128 µg/mL. The MIC_{90} of BG2 is 64 µg/mL fluconazole in VSM with 0 and 3 g/L acetate, and 128 µg/mL fluconazole in VSM with 1 and 2 g/L acetate.

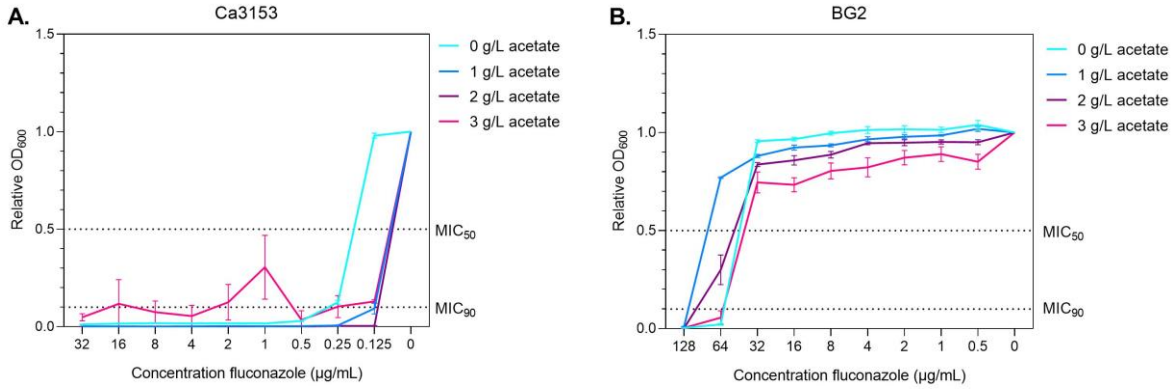


Figure 13: MIC curves of Ca3153 (A) and BG2 (B) in VSM with different combinations of fluconazole and acetate. Data are expressed as the average of four biological repeats ± SEM. MIC_{50} and MIC_{90} are indicated by horizontal dotted lines.

4.1.3.2. Fractional inhibitory concentration (FIC) indices indicate no synergism

To determine the combined effect of two compounds, normally a checkerboard assay is performed. This assay gives MIC values with which individual FIC values and an FIC index can be computed. When the FIC index is smaller than 0.5, there is synergy. When the FIC index is between 0.5 and 4, there is no significant difference between the combination and the individual effects of the two compounds. An FIC index bigger than 4 indicates antagonism. A checkerboard assay was performed to test the combined effect of fluconazole and acetate on the growth of *C. albicans* strain Ca3153 and *C. glabrata* strain BG2. The results are shown in Table 4. The FIC indices of Ca3153 both indicate no difference between the combined effect of fluconazole and acetate, and the individual effects of both compounds on the growth of Ca3153. The FIC_{50} index of BG2 also indicates no difference but the FIC_{90} index of BG2 is higher than 10 which indicates antagonism.

Table 4: Overview of results checkerboard assay and computed FIC index of fluconazole (FLC) and acetate for Ca3153 and BG2.

Ca3153			BG2		
Individual FIC values		**FIC index**	**Individual FIC values**		**FIC index**
FIC_{50} FLC	0.5	**1.42**	FIC_{50} FLC	1	**1.83**
FIC_{50} acetate	0.92		FIC_{50} acetate	0.83	
FIC_{90} FLC	1	**1.94**	FIC_{90} FLC	2	**> 10**
FIC_{90} acetate	0.94		FIC_{90} acetate	> 8	

4.2. *In vivo* experiments

In this part of the results, the vaginal mouse model was first optimized to obtain a stable *Candida* infection. After this was accomplished, a comparison was done between the effect of a probiotic *S. cerevisiae* strain and the antifungal drug fluconazole on mice with a vaginal *C. albicans* infection.

4.2.1. Direct injection of cell suspension results in a more stable infection compared to application with cotton swab

Usually, cotton swabs are used to obtain a vaginal *Candida* infection in mice. Due to the shortage of cotton swabs, mainly caused by the use of these cotton swabs in COVID-19 tests, another method needed to be found to infect the mice. For this reason, a mice experiment was done with in total 16 BALB/cJ mice to test the difference between a vaginal Ca3153 infection mediated by a cotton swab and a pipet. Different cell concentrations of *Candida* were also tested: $2.5x10^6$, 10^7 and 10^8 cells/mL. All the mice were injected with estradiol and infected with Ca3153. The timeline of the experiment can be seen in Figure 14 and the results are shown in Figure 15. The data in Figure 15 are expressed as the $\log_{10}$ of the colony forming units (CFU) of Ca3153 per mL vaginal fluid. At each lavage, vaginal fluid of the mice was collected and plated out. After two to three days the CFU were counted and from this the $\log_{10}$(CFU/mL) was calculated.

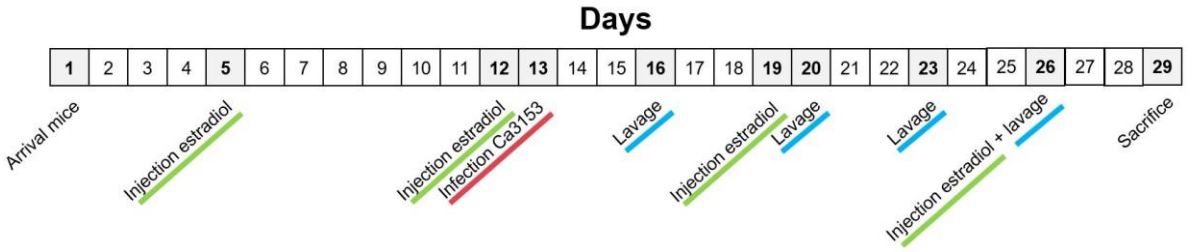


Figure 14: Timeline mice experiment: comparison direct injection with pipet and application with cotton swab. The handlings are indicated under the timeline at the day they were performed.

In Figure 15, the infection burden (CFU of Ca3153) is quite similar in the group infected by a cotton swab and by direct injection with $2.5x10^6$ cells/mL. The average of the $\log_{10}$ CFU values of these two groups are 2 to 3 $\log_{10}$ values lower compared to the other groups (pipet: $2.5x10^7$ and 10^8 cells/mL). The standard error of the mean (SEM) values in the groups infected by a direct injection with $2.5x10^7$ and 10^8 cells/mL are smaller compared to the other groups which indicates a more constant and stable infection over different mice. For this reason, $2.5x10^8$ cells/mL was chosen for the *Candida* infection of the last experiment.

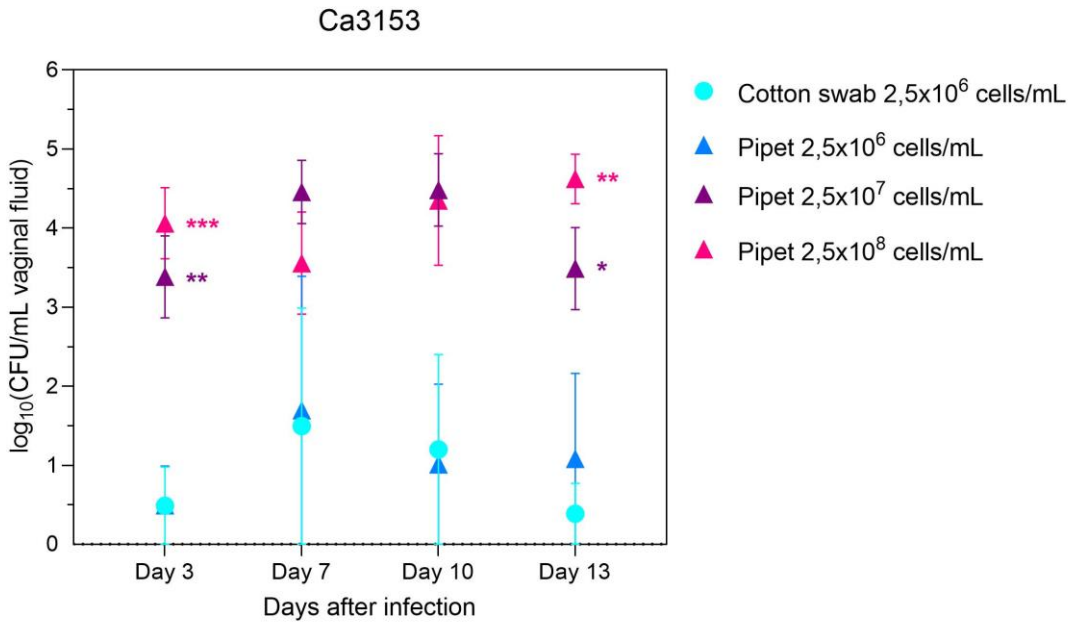


Figure 15: Colony forming units (CFU) of Ca3153 in vaginal fluid of BALB/cJ mice. Data are expressed as the average of four mice ± SEM. At day 3, 7, 10 and 13 after infection vaginal fluid was collected. The statistical significance was tested with Unpaired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a group infected with a pipet and the group infected with a cotton swab at that timepoint.
$P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

4.2.1.1. Probiotic treatment compared to fluconazole treatment

The first mice experiment where the effect of a probiotic *S. cerevisiae* strain was tested in this study (data not shown) failed because of the use of a different mice substrain: BALB/cByJ. This strain was used because the mice strain that is normally used (BALB/cJ) was out of stock. No conclusions could be made from the results of this experiment because there was an overreaction of the mouse immune system on the estradiol injections. The vaginas of the mice were extremely swollen, red and inflamed. Several mice were also euthanized before the end of the experiment. Because of this, only BALB/cJ were used in all other mice experiments.

In the second mice experiment where a probiotic treatment was tested, the effect of probiotic *S. cerevisiae* strain 6886 was compared to the effect of fluconazole on a vaginal Ca3153 infection. In total, 18 BALB/cJ mice were used divided in six groups. All the mice were injected with estradiol and infected with Ca3153. During treatment, 1X PBS was used as a vehicle application for the two control groups. Three groups received only one treatment (day 1 after infection) and the other three groups were treated three times (day 1, 7 and 14 after infection). The timeline of the experiment can be seen in Figure 16 and the results are shown in Figure 17. The data in Figure 17 are expressed as the $\log_{10}$ of the colony forming units (CFU) of Ca3153 or 6886 per mL vaginal fluid.

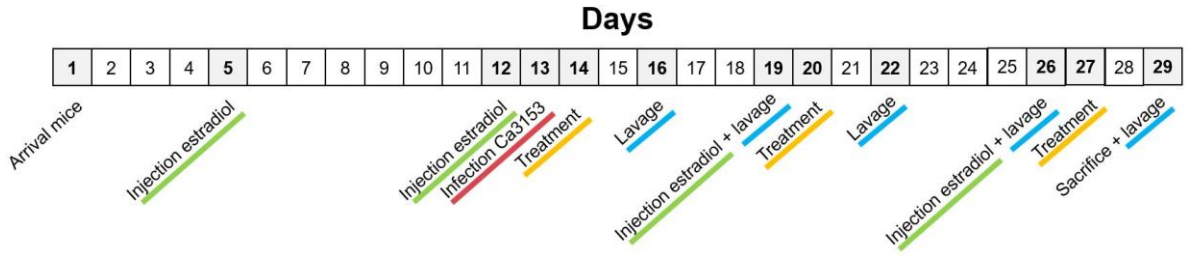


Figure 16: Timeline mice experiment: comparison probiotic and fluconazole treatment. The handlings are indicated under the timeline at the day they were performed.

In Figure 17A it can be seen that, at the first time point, three days after infection, the infection burden (CFU of Ca3153) is significantly lower for the probiotic treated group compared to the control group or the group treated with fluconazole. Over the course of time, however, as no additional treatment was applied, this effect diminishes completely. The infection burden in the fluconazole treated group is at all time points comparable to the control group except at timepoint 16, where it is significantly higher. There are no significant differences in Figure 17B. For the probiotic group, it seems that after every treatment the infection burden of Ca3153 drops a bit. A few days later however, it increases again. The difference in infection burden of Ca3153 between the control and the probiotic group is smaller immediately after the second and third treatment compared to the first treatment (Figure 17A and B). The infection burden in the repeated probiotic and fluconazole group are always the same as or lower than the control. In Figure 17C it can be seen that, two days after treatment, the $\log_{10}$ CFU of 6886 is 4 in the group that received a single treatment. From the next timepoint to the end of the experiment, no 6886 colonies were present anymore in the vaginal fluid of this group. In the group that received multiple treatment, 2.5 – 5 $\log_{10}$ CFU of 6886 was present at two days after a treatment. Three to four days later, the number of 6886 colonies decreased substantially just like in the group that received a single treatment.

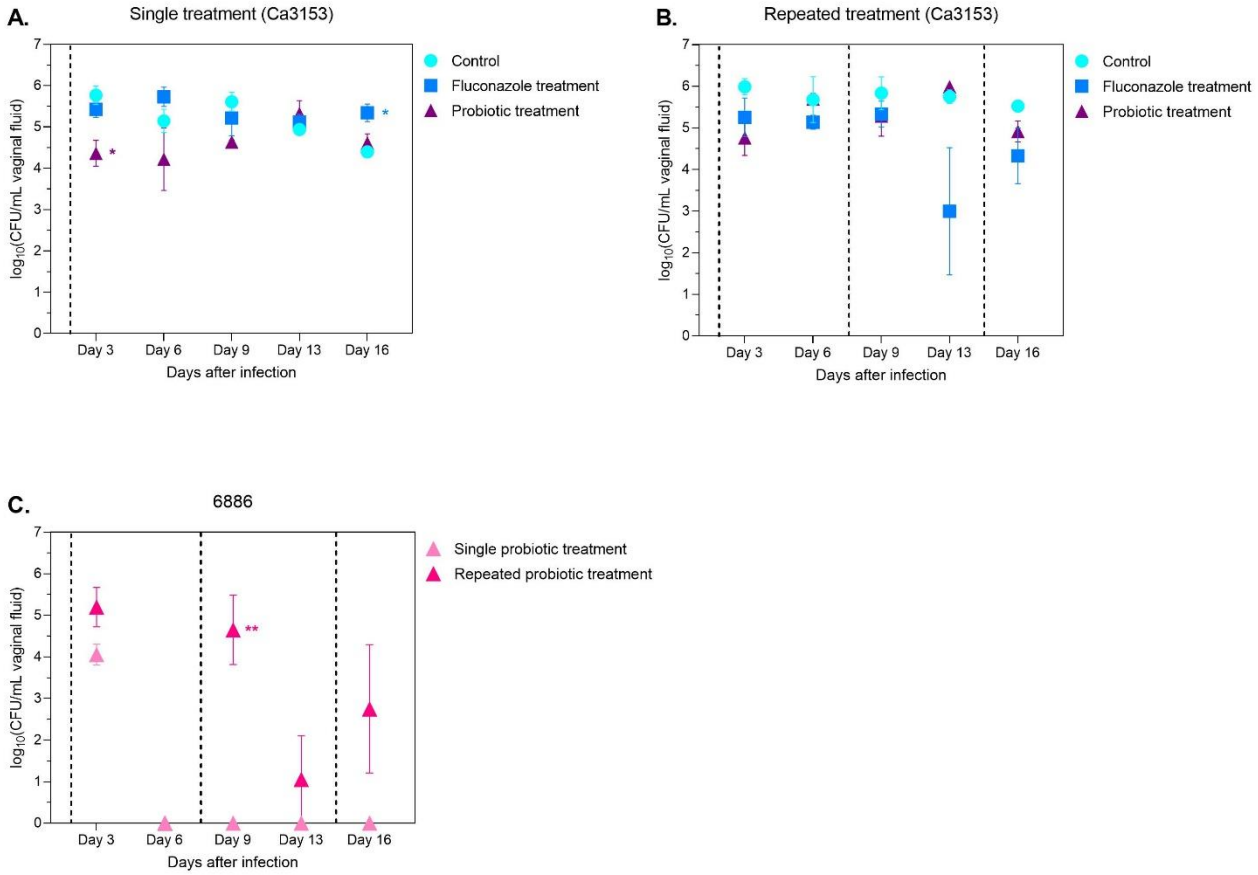


Figure 17: Colony forming units (CFU) of Ca3153 (A, B) and 6886 (C) in vaginal fluid of BALB/cJ mice. **(A)** shows the CFU of Ca3153 from the mice that received a single treatment (day 1 after infection). **(B)** shows the CFU of Ca3153 from the mice that were treated three times (day 1, 7 and 14 after infection). **(C)** shows the CFU of 6886 from the mice that were treated with a probiotic 6886 treatment. Data are expressed as the average of three mice ± SEM. At day 3, 6, 9, 13 and 16 after infection vaginal fluid was collected. The vertical dotted lines indicate the administration of a treatment. The statistical significance in **(A)** and **(B)** were tested with Unpaired one-way ANOVA tests with Bonferroni correction. An asterisk indicates a significant difference between a treatment group and the control at that timepoint. The statistical significance in **(C)** was tested with Unpaired t-tests with Bonferroni correction. An asterisk indicates a significant difference between the group that was treated once and three times. $P < 0.05$ (*), $P < 0.005$ (**), $P < 0.001$ (***)

5. DISCUSSION

A lot of women worldwide suffer from VVC and RVVC (Sobel, 2007). Besides the direct symptoms like more vaginal discharge, sexual dysfunction and physical pain, it can also cause serious complications during pregnancy (Gonçalves *et al.*, 2016). Pregnant women are more frequently colonized by *Candida* compared to non-pregnant women and these vaginal fungal infections are associated with a higher risk of premature labor and miscarriage (Holzer *et al.*, 2017). In more and more clinical studies drug-resistant *Candida* species are being recovered from women with VVC and RVVC (Whaley *et al.*, 2017; Yassin *et al.*, 2020). It has also been demonstrated that most women suffering from fungal infections caused by resistant *Candida* species have previously used antifungal drugs (Lírio *et al.*, 2019). The goal of this project is therefore to find a probiotic treatment of VVC/RVVC to reduce the general use of antifungal drugs and thus also the development of drug-resistant *Candida* species in women with VVC/RVVC. Especially for women with RVVC, that need to take antifungal drugs several times per year and that often experience recolonization with *Candida* within only one month after the end of their therapy, a probiotic treatment might allow them to remain infection-free for a longer period of time (Sobel *et al.*, 2001).

In the first part of this study, the effect of different fatty acid metabolites on the growth of *C. albicans* and *C. glabrata* was tested. We focus on *S. cerevisiae* as a potential probiotic and are thus most interested in the effect of SCFA directly or indirectly produced by *S. cerevisiae*, i.e. acetate, propionate and butyrate. The effect of lactate was also tested because the major end product of *Lactobacillus* species is lactate (Redondo-Lopez *et al.*, 1990). Many probiotics today contain lactobacilli (Bermudez-Brito *et al.*, 2012). Although it is proven that these bacteria can inhibit *Candida*, the role of lactate in their antifungal effect remains uncertain. The second part focusses on the azole susceptibility of *Candida* species and the possible synergy between azoles and acetate. Synergy between existing antifungal drugs and metabolites from a probiotic strain could be very beneficial in the treatment of *Candida* species that are less susceptible to antifungal drugs alone. The last part of this study deals with the *in vivo* optimization of a mouse model for vaginal *Candida* infections. Using the optimized model, we compared fluconazole-based treatment of VVC with a *S. cerevisiae*-based probiotic treatment. It is important that *in vitro* experiments are validated with *in vivo* results because more variables are included, the real-life niche is better simulated and the effect of the probiotic can be seen on the whole organism so that no side-effects on other organs are overlooked.

5.1. Effect of fatty acid metabolites on *Candida* growth

Growth assays were performed to test the effect of acetate on clinical isolates of *C. albicans* and *C. glabrata*. *S. cerevisiae* produces acetate during fermentation and the rate of production is strain-dependent (Franco-Duarte *et al.*, 2017). So, in case acetate appears to inhibit *Candida* growth in the vaginal niche, this could potentially be used as a selection criterium of *S. cerevisiae* strains during the development of probiotics. In this study, acetate inhibited the growth of all *Candida* species and isolates that were tested (Figure 5, 6 and 8). The inhibitory effect was concentration-dependent. Some strains were more resistant and needed a higher concentration to be inhibited. A concentration of 5 g/L acetate was enough to totally inhibit the growth of all *Candida* strains. This is in line with the findings of Lourenco *et al.* (2018). They found that a concentration of 4.5 g/L acetate is enough to inhibit both *C. albicans* and *C. glabrata* at a pH of 4 in both aerophilic and in microaerophilic conditions. This is an important conclusion since the vagina is a microaerophilic environment (Medina-Colorado *et al.*, 2017). They also found that the inhibiting effect of acetate on *Candida* increases when the pH becomes more acidic (Lourenco *et al.*, 2018). This is probably the case, because the more the pH drops, the more molecules of acetate exist in the undissociated form. It has been demonstrated that SCFA only exert their antifungal effect in undissociated form (Aldunate *et al.*, 2015; Guinan *et al.*, 2019; Lai *et al.*, 2009; Lourenco *et al.*, 2018). In our study, the effect of propionate and butyrate on *C. albicans* and *C. glabrata* was also tested in growth assays. *S. cerevisiae* itself cannot produce these acids, yet from various studies, it is known that it can stimulate their production in other microorganisms (Moens *et al.*, 2019; Schneider *et al.*, 2005). Both propionate and butyrate inhibited the growth of *C. albicans* strain Ca3153 and *C. glabrata* strain BG2 (Figure 11). The effect was concentration-dependent, similar to acetate. A concentration of 2 g/L butyrate completely inhibited the growth of both species. For propionate, 1 g/L was enough to completely inhibit the growth of only Ca3153 yet not BG2. This is not surprising as *C. glabrata* is intrinsically more resistant to a lot of compounds. These results are conform to the findings of Nguyen *et al.* (2011) and Yun and Lee (2016), who concluded that butyrate and propionate have an antifungal effect on *Candida* species. To use the stimulated production of propionate and butyrate in the development of new vaginal probiotics it is important to know which microorganisms are exactly stimulated by *S. cerevisiae* and if these species are sufficiently abundant in the human vagina to have an effect. If this is investigated and confirmed, experiments can be done to find which strains stimulate these microorganisms the most. So, in the future this could also become a criterium in the selection of the best anti-*Candida* strains.

The inhibiting effect of acetate, propionate, butyrate and other SCFA on *Candida* is shown to be concentration-dependent not only here but also in other studies (Guinan *et al.*, 2019). As previously stated in 1.4.2.4, the theories that are being proposed to explain the antifungal effect of SCFA are intracellular acidification, accumulation of anions, ATP depletion and perturbation of the plasma membrane (Bracey *et al.*, 1998; Guldfeldt & Arneborg, 1998; Mollapour *et al.*, 2008; Stratford & Anslow, 1998; Ullah *et al.*, 2012). The effects that acetate, propionate and butyrate exerted on the growth of *Candida* in the *in vitro* experiments of this study cannot be due to a change in environmental pH levels because the pH of the medium was adjusted to 4.2 after the SCFA were added. Even if the pH was not adjusted, their growth inhibiting effect would still unlikely be due to a change in environmental pH levels. *C. albicans* is capable of actively neutralizing acidic environments so SCFA-induced changes in environmental pH could not significantly affect their growth (Fan *et al.*, 2015; Guinan *et al.*, 2019; Vylkova *et al.*, 2011). Moreover, *C. albicans* and *C. glabrata* both have a broad external pH tolerance. They can survive in environments that have a pH between 2 and 10 (Sherrington *et al.*, 2017). In Rane *et al.* (2019), it was also shown that *C. albicans* cells with very acidic cytosols (pH ≤ 5.5) only show minimal growth defects, so their external pH tolerance can potentially be explained by their broad cytosolic pH range. SCFA do thus probably not exert their inhibitory effect on *Candida* species by intracellular acidification alone. To understand how weak organic acids exactly inhibit *Candida* cells on a molecular level, Cottier *et al.* (2015) looked at genomic and transcriptomic changes caused by lactate, acetate, propionate and butyrate. They found that each organic acid triggers the expression of unique combinations of genes. Despite the differences in the induced responses, all the organic acids regulated the same sixteen genes at all time points and independent of the pH. Thirteen of these genes (*CFL2*, *MP65*, *PIR1*, *ASR1*, *FET3*, *DAG7*, *GDH3*, *COI1*, *FRP1*, *6311*, *ICL1*, *FTR1*, *CAN1*) were up-regulated and three genes (*RPL13*, *HSP90*, *FTR2*) were down-regulated (Cottier *et al.*, 2015). There are large similarities with the transcriptional response to reactive oxygen species and seven out of sixteen genes (*CFL2*, *COI1*, *FRP1*, *PIR1*, *FET3*, *FTR1*, *FTR2*) are involved in the regulation of iron homeostasis (Chen *et al.*, 2011; Cottier *et al.*, 2015; Lan *et al.*, 2004). Cottier *et al.* (2015) also found that all weak organic acids decreased the intracellular iron levels of *C. albicans* cells by approximately 60%. However, restoring normal intracellular iron levels by using a mutant that imports more iron did not significantly affect the inhibitory effect of butyrate. So, the growth inhibition caused by lactate, acetate, propionate, and butyrate in their results cannot be explained by an intracellular iron drop alone. Still, iron is an essential micronutrient for *C. albicans* (Ramanan & Wang, 2000). It is critical for its growth, competition with other microbiota and interaction with the host (Purschke *et al.*, 2012; Ramanan & Wang, 2000). Hence, the fact that weak organic acids decrease the availability of this important micronutrient calls for further research.

Besides iron homeostasis, weak organic acids also had an effect on the expression of genes involved in host interaction, glycolysis, the biosynthesis of ATP, ergosterol, arginine and RNA and the biogenesis of ribosomes (Cottier *et al.*, 2015). All acids down-regulated genes involved in RNA synthesis and ribosome biogenesis, especially during longer exposure. This caused a significant reduction in total RNA and the ratio ribosomal RNA/total RNA in *C. albicans* cells (Cottier *et al.*, 2015). An overall down-regulation of transcription and translation is very typical for stress responses in general, and can be seen across a variety of microbial species (Lempiäinen & Shore, 2009). These data suggest that weak organic acids push *C. albicans* cells in a metabolic state similar to starved cells, in which the rates of transcription, translation and growth are low (Uppuluri & Chaffin, 2007).

In contrast to the results of acetate, propionate and butyrate, the results of lactate on *Candida* growth were not univocal in different experimental setups (Figure 5-7). The growth assays in the glass tubes indicated a stimulating effect of increasing concentrations of lactate on growth of both *Candida* species, while when growth was assessed in the 96-well setup, no effect was visible. Because *Candida* species are so metabolically versatile, they can also use lactate instead of their more-preferred carbon source glucose (Childers *et al.*, 2016). During their growth in VSM they will first mostly use glucose and then start metabolizing lactate. This probably explains the stimulating effect that lactate exerts on *Candida* growth. A possible reason why this stimulating effect is not visible in the 96-well setup is that the stationary phase of *Candida* growth is reached earlier and at a lower OD_{600} value in the 96-well setup compared to the glass tubes. The stationary phase is probably reached before the fungal cells started using lactate as carbon source. Thus, when the non-carbon nutrients are depleted, *Candida* cells are forced to enter the stationary phase without getting the chance to metabolize lactate. In Lourenco *et al.* (2018) the effect of lactate on *Candida* was also tested in both aerophilic and microaerophilic conditions. They saw no difference in the results which suggests that the difference between the results in the glass tubes and the 96-well setup in this study is probably also not due to a difference in oxygen availability. So, our tentative conclusion is that lactate stimulated *Candida* growth in both setups, but it is just not visible in the 96-well setup. To confirm this, the same experiments could be done with lower concentrations or no glucose in the VSM so that *Candida* cells have the chance to use lactate before the stationary phase is reached.

We hypothesized that the lack of inhibition by lactate compared to SCFA in various studies, including this study, might be due to the fact that the inhibition of lactate is pH-dependent like Lourenco *et al.* (2018) demonstrated with acetate. The pKa of lactate is 3.86, while the pH of the VSM is 4.2. At this pH, about 30% of the lactate molecules will be protonated while for acetate (pKa = 4.76) this is approximately 70%.

The results of the growth assays at varying pHs showed that lowering the pH from 4.2 to 3 has no influence on the effect of lactate on *Candida* (Figure 9). *Candida* species can mobilize lactate and SCFA even when glucose is present so a possible explanation for the lack of inhibition of lactate compared to SCFA could be that lactate is rapidly metabolized while the metabolization of SCFA may occur much more slowly giving them the time to exert their inhibitory effects (Childers *et al.*, 2016; Lourenco *et al.*, 2018). More research is needed to find the reason of the varying stimulating and inhibitory effects of lactate seen in different studies. But, since the lack of inhibitory effects of lactate is a conclusion of more and more studies, it also becomes increasingly clear that the production of lactate is probably not the most important mechanism by which *Lactobacillus* species inhibit *Candida*. Parolin *et al.* (2015) also reported that the amount of lactate in the supernatants of vaginal lactobacilli cultures do not correlate with the anti-*Candida* potential of the supernatants. Hence, lactobacilli probably also produce other inhibiting compounds.

Not all *Lactobacillus* species are able to produce both the L- and D-isomer of lactate. *L. iners* only has genes coding for L-lactate dehydrogenase in its genome, while *L. crispatus*, *L. gasseri* and *L. jensenii* have genes encoding both enzymes. *Lactobacillus* species also differ in the amount of lactate they produce and even within the same species, metabolic output can vary between strains. In addition to this, their effect on *Candida* can also differ between species (Papagianni, 2012; Witkin *et al.*, 2013). For this reason, growth assays were performed to find out if there is a difference in the effect of L- and D-lactate on *Candida* growth (Figure 10). Although no significant differences were found between 5 g/L D- and L-lactate, a slight trend can be seen that especially D-lactate has a stimulating effect on *Candida*. As previously explained, the effect of lactate differs in various studies (Kasper *et al.*, 2015; Krasner *et al.*, 1956). In a study from Oerlemans *et al.* (2020), the lactate production of several individual lactobacilli strains was evaluated. The different strains showed a wide production range from 2.78 g/L (*L. parabuchneri* AB17) to 20.22 g/L (*L. pentosus* KCA1). *L. rhamnosus* GG produced the highest amount of L-lactate (17.27 g/L) while *L. plantarum* WCFS1 produced the highest amount of D-lactate (7.68 g/L). Additionally, the latter strain inhibited *C. albicans* growth the most, implying that high amounts of D-lactate cause the greatest *C. albicans* growth inhibition (Oerlemans *et al.*, 2020). Although Oerlemans *et al.* (2020) suggested that a higher production of D-lactate is associated with a greater *Candida* inhibition, they did not directly test the effect of lactate on *Candida* growth. The greater inhibiting properties of the *L. plantarum* WCFS1 strain could also be the result of other antifungal mechanisms. As previously stated, our findings together with other studies indicate that the probiotic effect of certain *Lactobacillus* strains is probably not only due to the production of lactate. The differences between the effects of lactate in different studies can only be explained once the true mode-of-action is found.

5.2. Azole susceptibility and synergy with acetate

The azole susceptibility of *C. albicans* and *C. glabrata* was verified in this study, using broth microdilution assays to determine the minimally inhibitory concentration. Fluconazole was chosen because it is currently the most frequently used and preferred treatment for VVC caused by *C. albicans* (de Oliveira Santos *et al.*, 2018). The MIC_{50} of Ca3153 and BG2 are respectively 1 µg/mL and 128 µg/mL fluconazole. The MIC_{90} of Ca3153 is also 1 µg/mL while that of BG2 is more than 128 µg/mL (Figure 12). This corresponds to what was expected and what other studies found. In most studies the MIC values of *C. glabrata* are lower than 128 µg/mL, so BG2 is quite a resistant strain (Pfaller *et al.*, 2006; Yüksekkaya *et al.*, 2011). As explained in 1.2.3.2, drug tolerance is one of *C. glabrata*'s most important virulence factors. It is therefore no surprise that higher concentrations are needed to inhibit its growth compared to *C. albicans*. It is for this reason that women suffering from VVC/RVVC caused by *C. glabrata* are not treated with azoles but with amphotericin B in combination with boric acid (Sobel *et al.*, 2001). When the results of different studies are compared, the MIC values of fluconazole can vary between different strains of the same species but the concentration that is needed to inhibit *C. glabrata* strains will always be higher than *C. albicans* (Pfaller *et al.*, 2006).

As more and more *Candida* species are being identified with resistance to fluconazole and/or other antifungal drugs in women with VVC it is very relevant to test whether the metabolites of a potential probiotic strain possibly work synergistically with these drugs (Whaley *et al.*, 2017; Yassin *et al.*, 2020). Another reason for the importance of this test, is the observation that the effectiveness of antifungal drugs, including fluconazole, is lower at an acidic pH. Higher concentrations of fluconazole are needed to inhibit the growth of *Candida* species at pH 4 compared to pH 7. Most standardized MIC tests for antifungal drugs are done at pH 7, which gives misleading information about the effectiveness and needed concentrations of the drugs in acidic host environments, like the vagina (Danby *et al.*, 2012; Lourenco *et al.*, 2018). The exact mechanism behind the pH-reduced susceptibility of *Candida* species to antifungal drugs is not known yet. For all the above reasons, the combination of acetate and fluconazole was tested in this study on the growth of both *C. albicans* and *C. glabrata* with a MIC test and a Checkerboard assay (Figure 13 and Table 4). While the MIC test indicated a synergistic effect on *C. albicans*, no combined effect was seen on *C. glabrata*. The Checkerboard assay also indicated no synergistic effect and even an antagonistic effect on *C. glabrata*. However, several other studies did find synergism between acetate and fluconazole on the growth of both *C. albicans* and *C. glabrata* (Lourenco *et al.*, 2018; Moosa *et al.*, 2004). Undissociated organic acids, including acetic acid, can cause perturbation of the fungal plasma membrane, which makes the entry of azoles into *Candida* cells more easy (Mira *et al.*, 2010). Fluconazole is a fungistatic drug but Moosa *et al.* (2004) found that the combination of acetate and fluconazole has a fungicidal effect on *C. albicans*.

Since the MIC test (Figure 13A) indicates synergism for *C. albicans* but not for *C. glabrata* (Figure 13B) and neither did the Checkerboard assay (Table 4), maybe something technical went wrong during the preparation of these tests considering they were only performed once. Also, there are no major differences between the experimental setup used by Lourenco *et al.* (2018) and the one used in this study. So, a technical mistake is for now the only logical explanation. As several other studies have found a synergistic effect, it is certainly worth repeating these tests in the future (Lourenco *et al.*, 2018; Moosa *et al.*, 2004).

5.3. *In vivo* validation of *S. cerevisiae* probiotic treatment

The vaginal *Candida* mouse model was optimized to obtain a stable vaginal *Candida* infection. In previous vaginal mice experiments (data not shown), cotton swabs were used for application of the *Candida* cells to the vaginal cavity. A cotton swab was chosen because in the oral *Candida* mouse model, that was already optimized in this lab, this is also used to obtain an infection, so roughly the same protocol could be used for the vaginal model (Van Dyck *et al.*, 2020). However, due to the shortage of cotton swabs, mainly caused by the use of these swabs in COVID-19 tests, another method was needed to infect the mice. For this reason, a comparison was done between a direct injection of *Candida* cells in the vagina and an application with a cotton swab (Figure 15). In other studies that use the vaginal *Candida* mouse model, *Candida* cells are also directly injected in the vagina but without sedating the mice (Yano & Fidel, 2011). This way of vaginal infection was also previously tried in our lab but resulted in a very low vaginal *Candida* load (data not shown). A logical explanation for this is that the mice immediately started running around after the injection. The *Candida* cells did probably not get enough time to express the genes that are needed for adhesion to the vaginal epithelial cells, which resulted in a low fungal load. So, during the infection of this experiment the mice were sedated and placed on their back for one hour to allow the *Candida* cells to adhere to the vaginal epithelium. A direct injection of cells resulted in a more stable *C. albicans* infection compared to the application with cotton swabs. Different concentrations of *Candida* cells were also tested and a direct injection of $2.5x10^8$ cells/mL gave the highest vaginal fungal load (Figure 15). *Candida* species are not only able to adhere to biotic surfaces like epithelial cells and other fungal cells but also to abiotic surfaces like cotton swabs (Garcia *et al.*, 2011; Verstrepen & Klis, 2006). If the *Candida* cells adhere to the cotton swabs they will no longer adhere to vaginal epithelial cells. Cotton swabs are also porous, they act like sponges. This means that not all *Candida* cells are at the surface of the swab and there are also a lot of cells that are located deeper in the cotton swab. When the cotton swab is put in the vagina of a mouse, these cells are never in contact with the vaginal epithelium, so they do not get the chance to adhere to the vaginal wall. When the *Candida* cell solution is injected in the vagina, more cells are in contact with vaginal epithelial cells so more fungal cells will probably adhere.

Another explanation for the improved colonization using direct injection versus swabs, could be the anatomy of the mouse vagina. Just like in humans, not every mouse vagina has the same size. In some mice, the cotton swab barely fitted in their vagina, while in others there was quite a bit of room left. In mice with smaller vaginas, there was probably more contact between the swab and the vaginal epithelium compared to mice with wider vaginas. When you directly inject the *Candida* cell solution in the vagina, this will spread across the vaginal epithelium and the size of the vagina is less important. Thus, all of the above will probably have contributed to the result that a direct injection gives a more stable infection.

In the last experiment, the effect of probiotic *S. cerevisiae* strain 6886 was compared to the effect of fluconazole on a vaginal *Candida* infection in mice (Figure 17). Strain 6886 was chosen based on preliminary data of colleagues Mart Sillen and Jade Michiels. *In vitro*, 6886 was the best overall performing *S. cerevisiae* strain in inhibiting *Candida* across the following categories: inhibition of adhesion, filamentation and growth. The last couple of years the interest in probiotics against *Candida* infections is growing fast. This is partly due to the concerningly high number of resistant *Candida* isolates that are continually being recovered, not only from women with VVC or RVVC but also other clinical patients (Whaley *et al.*, 2017; Yassin *et al.*, 2020). When patients are diagnosed with fungal infections, antifungal drugs are quickly prescribed without paying attention whether these patients already took these drugs in the past. If the extensive and repeated use of antifungal drugs does not decrease in the future, by exploiting alternatives like probiotics, a fungal-resistance crisis will develop in which *Candida* isolates will no longer be susceptible to any antifungal drugs. The results of the comparison between a probiotic *S. cerevisiae* treatment and fluconazole are very promising (Figure 17). Two days after a probiotic treatment, the number of *Candida* cells retrieved through lavage is lower than the control and even lower than the fluconazole-treated group. A few days later however, this effect diminishes slowly. The disappearance of this inhibitory effect of strain 6886 can be explained by looking at Figure 17C. Here it can be seen that two days after treatment, *S. cerevisiae* colonies are present in the vaginal fluid of the mice but three to four days later the number of colonies decreases until none are present anymore. For the future this means that if strain 6886 is ever chosen for the development of a probiotic, it will likely need to be administered every few days to prolong the inhibiting effect. If a probiotic is preferred that only needs to be administered once or twice, screening should be done for strains that are better able to adhere to the vaginal wall. This adhesion ability can easily be tested *in vitro* using HeLa cells.

The inhibitory effect of the second and third dose of probiotic strain 6886 seems less pronounced compared to the first dose (Figure 17A and B). This could be due to the pH of the buffer solution (1X PBS) used throughout the experiment. The *S. cerevisiae* cells and fluconazole were dissolved in this buffer solution for treatment and also during the lavages it was used. The pH of a mouse vagina is nearly neutral and ranges between 5 and 8 (Balaci *et al.*, 2009; Mahadevan & Balamuthu, 2016). The buffer solution has a pH of 7.4 and will make the vaginal pH of the mice even more neutral than it already is. Also, *S. cerevisiae* produces fatty acid metabolites that could potentially lower the vaginal pH but regularly adding this buffer solution will counteract this effect. Thus, at the beginning of the experiment, when the buffer solution was not yet added multiple times, the pH of the vagina was perhaps slightly lower than it was towards the end of the experiment. A suggestion for future repetitions of this experiment is to use saline instead of a buffer to dissolve the drug and suspend the cells. The inhibiting effect that probiotic strain 6886 exerted on the growth of *Candida* in the mice is probably not due to the production of fatty acid metabolites. As previously mentioned, the pH of a mouse vagina is nearly neutral (Balaci *et al.*, 2009; Mahadevan & Balamuthu, 2016). As the pKa of acetate and succinate are respectively 4.76 and 4.21, they will probably mostly exist in anion form in the mouse vagina. They will still be able to get in the *Candida* cells by monocarboxylate transporter Jen1 and dicarboxylate transporter Jen2 but they will no longer exhibit an antifungal effect (Vieira *et al.*, 2009). It has been demonstrated that in anion form, fatty acid metabolites no longer exhibit inhibitory effects (Aldunate *et al.*, 2015; Guinan *et al.*, 2019; Lai *et al.*, 2009; Lourenco *et al.*, 2018). There are many other mechanisms by which strain 6886 could have possibly inhibited *Candida*. Some examples of mechanisms by which *S. cerevisiae* strains were demonstrated to inhibit *Candida* are inhibition of adhesion by suppressing the expression of Sap genes, inhibition of germ-tube formation and inhibition of filamentation (Pericolini *et al.*, 2017). The inhibiting effects of the fluconazole treatments are less pronounced as expected and at certain timepoints the infection burden is even higher than the control group (Figure 17). Maybe a concentration of 200 µg/mL is just not enough to affect the growth of *C. albicans* strain Ca3153 *in vivo*. So, this concentration could be increased for future experiments. It is certain that there is nothing wrong with the fluconazole compound used in the experiment because the same batch was used during the *in vitro* experiments in which fluconazole clearly inhibited *Candida*. Since several other studies administered fluconazole orally to treat vaginal *Candida* infections in mice, this administration route could also be tested in future experiments (González *et al.*, 2009; Mikamo *et al.*, 1995). Fluconazole has a moderate lipophilicity and a poor water solubility which results in a low skin penetration (Vlaia *et al.*, 2021). Although, topical fluconazole treatments are less common due to these physicochemical properties, they exist and are proven to be effective (Muñoz *et al.*, 2020; Yim *et al.*, 2010). Thus, the reason why the intravaginal fluconazole treatment did not give the expected results remains a question.

Current research on vaginal *S. cerevisiae*-based probiotics is not focused on the beneficial effects of fatty acid metabolites. The combination of the more neutral vaginal pH of almost all mammals (except humans) and the fact that fatty acid metabolites only exert inhibiting effects in acidic environments could partly explain this (Lourenco *et al.*, 2018; Miller *et al.*, 2016). There is currently no *in vivo* model available that has the same vaginal pH as humans. To research and exploit the fatty acid mode-of-action of *S. cerevisiae* strains, maybe a new mouse model needs to be developed that includes mice with a more acidic vagina. Human microbiota-associated (HMA) mice could be the solution. A HMA mouse model of the human vaginal microbiota in which human vaginal microbiota are transferred to the vaginas of germ-free mice already exists (Wolfarth *et al.*, 2020). Since lactate-producing lactobacilli are the main cause of the acidic vaginal pH in humans, the pH of the vagina of HMA mice with human vaginal microbiota will probably also be more acidic (Miller *et al.*, 2016). Another advantage of this model is that all the interactions between the vaginal microorganisms that happen in humans, can also be simulated. Another relevant option, that would also reduce the number of laboratory animals in research, is an *in vitro* organ-on-a-chip model of the human vagina (Mancini & Pensabene, 2019). It consists of a three-dimensional microfluidic cell culture chip that simulates the conditions of a specific organ. Vaginal epithelial cells, mucus, immune cells and microbiota of women with VVC/RVVC or healthy women can all be added to this system. Parameters like for example the pH can also easily be controlled. Both the HMA mouse model and vagina-on-a-chip would be completer, more realistic and more relevant as vaginal model.

6. CONCLUSIONS

The aim of the ScerViCs project, of which this study is a part, is to develop a *S. cerevisiae*-based probiotic treatment of vulvovaginal *Candida* infections. The focus of this study was the production of fatty acid metabolites as potential probiotic mode-of-action. The three main objectives of this study were accomplished and the results look very promising for the future of the ScerViCs project.

The first objective was to investigate the effect of SCFA, directly or indirectly produced by *S. cerevisiae* (acetate, propionate and butyrate), on different *Candida* species in the vaginal niche. Acetate, propionate and butyrate had an inhibitory effect on the growth of both *C. albicans* and *C. glabrata* in vaginal simulative medium. A sub-objective was to compare the effect of these SCFA with the effect of lactate, the major end product of lactobacilli, on *Candida* in the vagina. In contrast to the SCFA, lactate turned out to have a stimulating effect on *Candida*. Neither lowering the pH well below the pKa of lactate, nor diversifying between L- and D-lactate showed to inhibit *Candida* growth. Our results suggest that the production of lactate is probably not the most important mechanism by which *Lactobacillus* species inhibit *Candida*.

The second objective was to investigate the combination effect of fluconazole, the most used antifungal drug in the treatment of vaginal fungal infections, and acetate on *Candida* in the vagina. First, the azole susceptibility of the *Candida* species was tested. *C. glabrata* was more resistant to fluconazole, so higher concentrations were needed to inhibit its growth compared to *C. albicans*. Our results for the combination effect were not univocal. One test indicated synergism for *C. albicans* but not for *C. glabrata*. Another test indicated no synergism for both species. Since other studies did find a synergistic effect between fluconazole and acetate, something technical might have gone wrong during the execution of these experiments in this study.

The third objective was to validate the effectiveness of a *S. cerevisiae*-based probiotic treatment in an *in vivo* mouse model for vaginal *Candida* infections. The vaginal mouse model was first optimized to obtain a stable vaginal *C. albicans* infection. A direct injection of a *C. albicans* cell suspension of 2.5×10^8 cells/mL resulted in the most stable vaginal infection with the highest fungal load. Using the optimized vaginal candidiasis mouse model, the effect of probiotic *S. cerevisiae* strain 6886 was compared to fluconazole. Two days after the probiotic treatment the vaginal load of *Candida* was lower than the untreated and fluconazole-treated group. The effect slowly diminished after a few days because the adhesion ability of the *S. cerevisiae* strain was probably not great. The inhibiting effect of fluconazole was less pronounced as expected.

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ADDENDUM

1. Risk Assessment

Doing research includes working with chemical substances and biological agents which involves potential risks for the health of the researcher and the environment. It is therefore important to assess and predict these risks to avoid them by taking precautions.

1.1. Risks related to chemical substances

When working with chemical substances, wearing personal protective equipment (PPE) such as a lab coat and gloves is obligated at all times. In the case of working with certain chemicals, extra precautions need to be taken. These are listed down below.

1.1.1. Ethanol

Ethanol with a concentration of 70% is always available in the lab to disinfect areas, gloves and materials that are contaminated. It is a highly flammable liquid that should never be close to any heat source. When using a Bunsen burner, it is important to first wait until the ethanol on your gloves has evaporated. Additionally, ethanol can cause irritation to the eyes and skin so it should be used with precaution.

1.1.2. Isoflurane

Isoflurane is a non-flammable, volatile anaesthetic used for the sedation of mice. It is administered by inhalation. It is important to tightly seal the cone around the animal's nose and keeping the anaesthetic chamber closed while the isoflurane tap is open to limit the exposure of the researcher to the gas. After prolonged exposure it can cause dizziness, a head-ache and shortness of breath. It is also considered as a potential carcinogenic substance.

1.1.3. Weak acids

Acetic acid is a highly flammable, corrosive liquid. It is stored in a fireproof storage cabinet. It is harmful in contact with skin and eyes and when it is inhaled or swallowed. It can cause severe skin and eye damage. Lactic acid is corrosive and can cause eye, skin, digestive and respiratory damage. Propionic acid is flammable and corrosive. It can cause eye, skin, digestive and respiratory tract burns. Butyric acid is combustible and corrosive. It can cause severe eye and skin damage. It is best when working with weak acids to use them under a chemical fume hood. Flammable/combustible substances are mandatorily only opened and used under a chemical fume hood far away from heat sources.

1.2. Risks related to biological agents

When working with biological agents, wearing personal protective equipment (PPE) such as a lab coat and gloves is obligated at all times.

1.2.1. *Candida* species

C. albicans and *C. glabrata* are classified as risk group 2 organisms. They are unlikely to be a significant risk to researchers in the lab or the environment, but exposure may cause infection. Working with *Candida* species requires an L2 lab (in which no windows can be opened and doors have to remain closed when not passing). The handling of the organisms is done under a laminar flow. Working areas need to be disinfected with 70% ethanol or Dettol®. All waste materials that were in contact with the agents need to be discarded in yellow biohazard boxes. Used glassware is first autoclaved and then washed before it can be reused. Gloves should regularly be disinfected using 70% ethanol and hands should be washed and disinfected with alcogel before leaving the lab.

1.2.2. *S. cerevisiae*

S. cerevisiae is classified as a risk group 1 organism. These microorganisms are unlikely to cause disease. It is allowed to work with it on an open bench but since a laminar flow is available in an L2 lab it is usually handled under a laminar flow.

1.3. Risks related to laboratory animals

1.3.1. Laboratory mice

A FELASA B certificate is required to conduct mice experiments. All mice experiments were approved by the ethical committee of KU Leuven. The mice were purchased from an approved distributor. This reduces the risk of zoonosis. They were kept in autoclaved filter-top cages in a room with Animal Biological Safety Level-2. In this room it is obligated to wear a lab coat, shoe covers and a hairnet. When handling the mice, wearing gloves is obligated. To reduce the risk of developing allergies, wearing a mouth mask is recommended. Waste is discarded in yellow biohazard boxes. When leaving the animal room, hands should be washed and disinfected. The cadavers of the mice are collected in biohazard box at -20°C. This is collected regularly by the authorized services of KU Leuven. In case of animal bites, a standard procedure of disinfection and reporting has to be followed.

1.4. Risks related to physical hazards

Needles, used during the mice experiments, are disposed in a special yellow sharps bin. UV-radiation was used to sterilize certain materials, like for example 96-well plates. Excessive short-term exposure can cause skin burns and increases the risk of skin cancer. It can cause acute eye damage, after prolonged exposure it can cause permanent eye damage. To avoid this, the UV-sterilizer should only be opened when the UV-lamp is off.

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