TÉCNICO LISBOA

UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Dissecting the molecular mechanisms underneath the interaction between human colonizing *Lactobacillus* and the pathogenic yeasts *C. glabrata* and *C. albicans*

Nuno Alexandre Aparício Pedro

Supervisor: Doctor Nuno Gonçalo Pereira Mira

Thesis approved in public session to obtain the PhD Degree in

Biotechnology and Biosciences

Jury final classification: **Pass with Distinction**

2023

UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Dissecting the molecular mechanisms underneath the interaction between human colonizing *Lactobacillus* and the pathogenic yeasts *C. glabrata* and *C. albicans*

Nuno Alexandre Aparício Pedro

Supervisor: Doctor Nuno Gonçalo Pereira Mira

Thesis approved in public session to obtain the PhD Degree in

Biotechnology and Biosciences

Jury final classification: **Pass with Distinction**

Jury

Chairperson: Doctor Arsénio do Carmo Sales Mendes Fialho, Instituto Superior Técnico, Universidade de Lisboa

Members of the Committee:

Doctor Gabriel António Amaro Monteiro, Instituto Superior Técnico, Universidade de Lisboa

Doctor Nuno Gonçalo Pereira Mira, Instituto Superior Técnico, Universidade de Lisboa

Doctor Isabel Antunes Mendes Gordo, Instituto Gulbenkian de Ciência, Fundação Calouste Gulbenkian

Doctor Mariana Contente Rangel Henriques, Escola de Engenharia, Universidade do Minho

2023

Abstract

The emergence of *Candida* species resistant to currently used antifungals is one of the more relevant factors determining the outcome of patients suffering from infections caused by these species pushing the need of developing new anti-*Candida* therapies and new targets different from those targeted by current antifungals. A hypothesis under the table is the use of probiotics to treat *Candida* infections. The human host is widely populated by a cohort of organisms defined as the human microbiota. Among these organisms, some *Lactobacillus* species are constantly isolated from the vaginal and intestinal tract, being considered endogenous to the human host. The constant observations that probiotics containing lactobacilli have positive outcomes for patients infected with *Candida* and that these bacteria can reduce the growth and virulence traits of *Candida in vitro* suggest a possibility of using lactobacilli based probiotics against these yeast infections. Thus, this thesis focused on the interaction of two lactobacilli species, *Lactobacillus gasseri* and *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), considered endogenous to the vaginal and intestinal tracts, respectively, with *Candida albicans* and *Candida glabrata*, two *Candida* species commonly isolated from these niches. This manuscript demonstrated that *L. gasseri* and *L. reuteri* were able to reduce growth and virulence traits of *C. glabrata* and *C. albicans* in planktonic and biofilm forming conditions, this effect being attributed to the presence of acetate, at least in *L. gasseri*. Additionally, we unveiled relevant genomic adaptations of *C. glabrata* and *L. gasseri*, including competitiveness genes of *C. glabrata* responsible for survival in co-cultivation using transcriptomics and phenomics analysis that included members of the HOG, PKA and calcineurin signalling pathways. In summary, the results discussed in this thesis are anticipated to enhance the development of new probiotic-based therapies, using endogenous lactobacilli species, for combating *Candida* infections, that could potentially alleviate the significant health burden posed by *Candida* infections in humans.

Key-words: *Candida*, *Lactobacillus*, transcriptomics, co-cultivation, competitiveness

Resumo

O aparecimento de espécies de *Candida* resistentes aos antifúngicos atualmente utilizados é um dos fatores mais relevantes que determinam o desfecho dos doentes que sofrem de infeções causadas por estas espécies, o que torna necessário o desenvolvimento de novas terapias anti-*Candida* e de novos alvos diferentes dos visados pelos antifúngicos atuais. Uma hipótese em estudo é a utilização de probióticos para tratar as infeções por *Candida*. O hospedeiro humano é amplamente povoado por um conjunto de organismos definidos como a microbiota humana. Entre estes organismos, algumas espécies de *Lactobacillus* são constantemente isoladas do trato vaginal e intestinal, sendo consideradas endógenas ao hospedeiro humano. As constantes observações de que probióticos contendo lactobacilos têm resultados positivos para pacientes infetados com *Candida* e que essas bactérias podem reduzir o crescimento e a virulência da *Candida in vitro* sugerem a possibilidade de usar probióticos à base de lactobacilos contra essas infeções. Assim, esta tese centrou-se na interação de duas espécies de lactobacilos, *Lactobacillus gasseri* e *Limosilactobacillus reuteri* (anteriormente *Lactobacillus reuteri*), consideradas endógenas dos tratos vaginal e intestinal, respetivamente, com *Candida albicans* e *Candida glabrata*, duas espécies de *Candida* comummente isoladas destes nichos. Esta tese demonstrou que *L. gasseri* e *L. reuteri* foram capazes de reduzir o crescimento e a virulência de *C. glabrata* e *C. albicans* em condições planctónicas e de formação de biofilme, sendo este efeito atribuído à presença de acetato, pelo menos em *L. gasseri*. Além disso, revelámos adaptações genómicas relevantes de *C. glabrata* e *L. gasseri*, incluindo genes de competitividade de *C. glabrata* responsáveis pela sobrevivência em co-cultura, utilizando abordagens de transcriptómica e fenómica, que incluíram membros das vias de sinalização HOG, PKA e calcineurina. Em suma, espera-se que os resultados discutidos nesta tese contribuam para o desenvolvimento de novas terapias baseadas em probióticos, utilizando espécies endógenas de lactobacilos, para combater as infeções por *Candida*, o que poderia potencialmente aliviar os encargos significativos para a saúde colocados por estas infeções nos seres humanos.

Palavras-chave: *Candida*, *Lactobacillus*, transcriptómica, co-cultivo, competitividade

Acknowledgements

First and foremost, I want to express my gratitude to Professor Nuno Mira, my supervisor, for his invaluable guidance, engaging scientific discussions, and for creating a supportive group environment. I am also thankful for the numerous opportunities provided to me, including the chance to learn new techniques and visit various laboratories. These experiences have been essential for the completion of this project and have greatly contributed to my personal and professional development.

In addition, I would like to express my appreciation to Professor Isabel Sá-Correia, the former head of the Biological Sciences Research Group (BSRG), and Professor Arsénio Fialho, the current head of the BSRG, for granting me the opportunity to collaborate with an exceptional team. Their leadership has been instrumental in creating an inspiring and productive research environment. Furthermore, I would like to acknowledge Professor Margarida Casal, the coordinator of the Doctoral Program in Applied and Environmental Microbiology (DP_AEM), for providing me with the chance to be a part of a vibrant and collaborative community of both students and professors.

I would like to express my gratitude to all the collaborators of the work presented in this thesis, with special mention to Dr Sarah Lebeer and her team at the University of Antwerp, especially Dr Dieter Vandenheuvel. I am immensely grateful for their warm reception during my experience abroad, which provided me with a unique opportunity to enhance my scientific knowledge. Additionally, I would like to extend my thanks to Dr Hiroji Chibana for generously providing some of the *Candida glabrata* mutant strains. I am also appreciative of the collaborative work with Dr Sónia Silva from the University of Minho, Dr Sandra Silva from the Institute for Bioengineering and Biosciences (iBB) and Dr Marta Alves from CQE-Centro Química Estrutural, Instituto Superior Técnico for their help on confocal and electronic microscopy analysis.

I acknowledge “Fundação para a Ciência e a Tecnologia” (FCT) PhD grant PD/BD/143026/2018 attributed to the candidate under the framework of the DP_AEM PhD program in Biotechnology and Biosciences. Additionally, I acknowledge the financial funding to iBB (project no. 007317) by FCT in the scope of the project UIDB/04565/2020 and the project LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy - i4HB. Finally, I acknowledge the project LactoCan (PTDC/BIA-MIC/31515/2017) received from FCT which was the backbone of this thesis.

I would like to extend my heartfelt gratitude to all the members of the remarkable "NPM group" and my colleagues from the BSRG who have contributed in various ways to my work and have accompanied me throughout this incredible journey. I want to express a special thanks to Dr Mónica Galocha, Dr Sara Salazar, Dr Ana Vila-Santa, Dr Pedro Pais, Dr Tiago Pita, Dr. Joana Feliciano, Maria João Tavares, Maria Joana Pinheiro, Joana Valério, Gabriela Fontebasso, Catarina Lima, Ana Paula and Mónica Rato for their support, assistance, and patience throughout

the entire process. Their presence and collaboration have been invaluable, and I am deeply grateful for their contributions.

Lastly, I want to express my deepest gratitude to my beloved partner, Sónia, for her boundless love, solid support, incredible patience, and unwavering belief in me. To my family, I extend my heartfelt appreciation, with special mention to my parents and younger sister. I have always been able to rely on their love and support throughout my life's journey. Words cannot fully convey how much I love and cherish each and every one of you.

List of acronyms

1-ABC	1-acetyl-β-carboline
3-HPA	3-hydroxypropionaldehyde
5-FC	5-flucytosine
5-FU	5-fluorouracil
5-FUMP	5-fluorouridine monophosphate
ABC	ATP-binding cassette
ALS	Agglutinin-like sequence
APF	Aggregation promoting factors
ATP	Adenosine triphosphate
BSI	Bloodstream infections
BV	Bacterial vaginosis
CDI	*Clostridium difficile* infection
cEPSs	Capsular exopolysaccharides
CFU	Colony forming unit
CHC	Chronic hepatitis C virus infection
COG	Cluster of ortholog genes
CSTs	Community state types
CTI	Chlamydia trachomatis infection
CVF	Cervicovaginal fluid
CVS	Cervicovaginal secretion
DEC	Diarrheagenic *Escherichia coli*
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
eDNA	extracellular DNA
EPSs	Exopolysaccharides
FdUM	5-fluorodeoxyuridine monophosphate
FUTP	5-fluorouridine triphosphate
GBA	Gut-to-brain axis
GDH	Glycerol dehydratase
GIT	Gastrointestinal tract
GO	Gene ontology
HBD	Human beta defensin
HCV	Hepatitis C virus infection
HePSs	Heteropolysaccharides
HICA	2-hydroxyisocaproic acid
HoPSs	Homopolysaccharides
HPI	*Helicobacter pylori* infection
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IECs	Intestinal epithelial cells
IECs	Intestinal epithelial cells
ITS	Internal transcribed spacer
IUDs	Intrauterine devices
LAPs	Linear azol(in)e-containing peptides
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MFI	Median fluorescence intensity
MFS	Major facilitator superfamily
MS	Mass spectrometry
MUBs	Mucus-binding proteins
MOPS	3-morpholinopropane-1-sulfonic acid
NACS	Non-albicans *Candida* species
NADPH	Nicotinamide adenine dinucleotide phosphate
PAMP	Pathogen-associated molecular patterns
PCA	Principal components analysis
pHi	Internal pH
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RHVE	Reconstituted human vaginal epithelium
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Rotation per minute
RVVC	Recurrent vulvovaginal candidiasis
SAP	Secreted aspartyl proteinase
SEM	Scanning electron microscopy
sEPSs	Slime exopolysaccharides
STIs	Sexually transmitted infections
STIs	Sexually transmitted infections
TLR	Toll-like receptor
VMB	Vaginal microbiota
VVC	Vulvovaginal candidiasis
WGS	Whole genome sequencing
WHO	World Health Organization

Table of Contents

List of tables

List of figures

I. Introduction

Part of the contents described in this chapter had been published in:

1. Lourenço A, Pedro NA, Salazar SB, Mira NP, "Effect of Acetic Acid and Lactic Acid at Low pH in Growth and Azole Resistance of *Candida albicans* and *Candida glabrata*", **Frontiers in microbiology** 9(3265), 2018;
2. Salazar SB, Simões RS, Pedro NA, Pinheiro MJ, Carvalho NFNN, Mira NP, "An overview on conventional and non-conventional therapeutic approaches for the treatment of candidiasis and underlying resistance mechanisms in clinical strains", **J Fungi**. 10;6(1), 2020;
3. Nuno A. Pedro and Nuno P. Mira, "An overview of the potential of vaginal and intestinal lactobacilli species to control infections prompted by *Candida* species and of the underneath molecular mechanisms" (submitted).

I.1. Overview

The idea that to function properly the human body depends not only on the way by which its highly specialized cells interact and communicate with one another, but also from their interaction with a vast cohort of microorganisms that co-colonizes the different niches, is a hot topic in nowadays research. This large set of microorganisms, along with their genetic content, is commonly known as the human microbiota and consists of millions of bacterial, archaeal, and small eukaryotic species along with viruses[1]. With the prominent advances in the development of metagenomics, it has been possible to show that these microbial communities are complex and comprise many more species than those first believed to serve as human colonizers. With this metagenomics-derived data it has been possible to estimate the abundance of these microbial populations as being around ten-fold higher than the abundance of human cells[2]. In this context, humans can be seen as holobionts, that is, “super-organisms” composed of a complex interplay between human cells and commensal microbes that co-evolved and established close relationships that shape homeostasis[3]. The composition of the individual microbiome is diverse and reflects not only traits associated with the genetic portrait of each individual, but also “environmental” factors, out of which diet has probably been the one most studied[4-6]. Evidence to date suggests that long-term diets shape the individual gut microbiome, however, a sufficiently extreme short-term dietary change can also cause prominent changes in the gut microbiomes and people sharing diets can show remarkable resemblances of their microbiomes within days[4-7]. The use of antibiotics has also been found to have a strong effect in modulating the gut microbiome with certain species being more affected than others, possibly due to differences in their growth phase or metabolic state[8]. Other important factors found to influence the microbiome include those that directly impact lifestyle such as sleep deprivation[9], stress[10], occupation[11], sexual activity[12], ownership of a pet[13], or regular physical exercise[14]. Despite these changes prompted by the environment in the overall microbiome composition, it is becoming increasingly clear that certain species or genera can establish colonization, being consistently found in different individuals of the same population. The consistent identification of these species may result from them having evolved specific adaptive responses that allowed them to thrive in these variable conditions, probably due to years of co-evolution in association with the host. In most cases, the molecular players underpinning these adaptive responses remain to be characterized, a reflection of the poor knowledge gathered on the biology and physiology of most of these indigenous human colonizing microbial species.

One of the aspects that has been more intensely scrutinized is how changes in the microbiome impact health, with evidences accumulating that the imbalance of microbial communities (a condition also known as dysbiosis) can lead to the dysregulation of bodily functions and development of heart, respiratory or liver diseases or cancer (reviewed in Kaijian Hou *et al.*, 2022) (Figure I.1)[15].

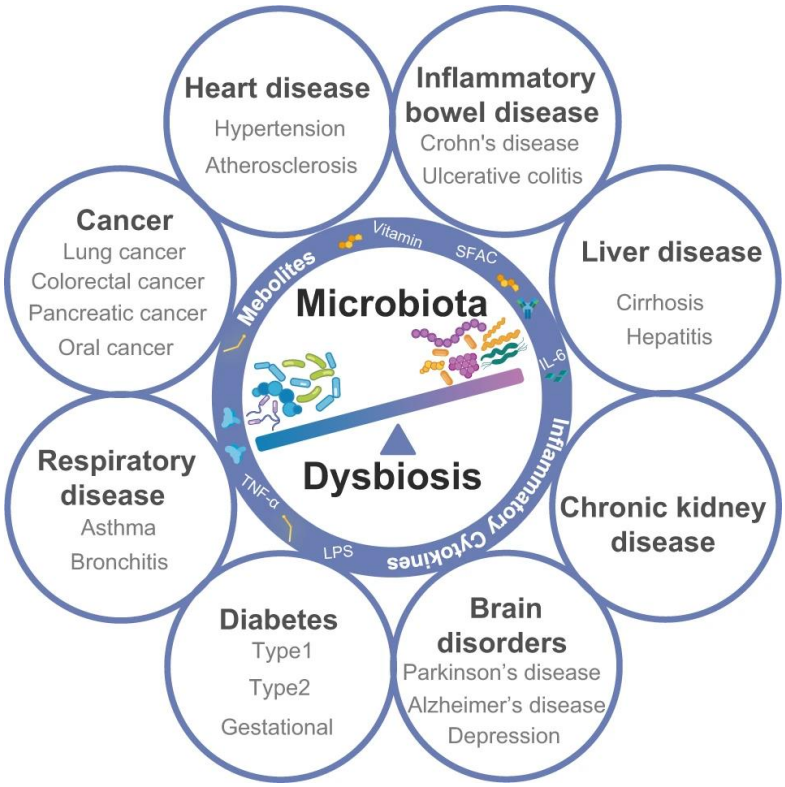


Figure I.1. The human microbiota contributes to the health of various human host niches. Reduction in the abundance of health associated microbiota species leads to a state of dysbiosis and the increased risk of developing diseases, including cancer, hypertension, inflammation bowel disease, diabetes, and asthma among many others. Figure taken from Kaijian Hou *et al.*, 2022[15].

This thesis will specifically address the potentially protective effect of *Lactobacillus* species in the vaginal (with emphasis on *L. gasseri*) and intestinal (with emphasis on *L. reuteri*) tracts. Thus, in the introduction, it is provided a general overview of the microbial diversity observed in the vaginal and intestinal tracts, after which are described relevant aspects of physiology, molecular biology, and genomics of *L. gasseri* and *L. reuteri* due to the central role of these species in this thesis. The focus of the project is the exploration of the lactobacilli species in preventing infections caused by yeasts of the *Candida* genus, significant vaginal pathogens. Consequently, aspects related to the pathogenesis of infections caused by *Candida* (with a focus on vaginal candidiasis), as well as the state-of-the-art regarding the use of probiotics for the treatment of vaginal candidiasis will be addressed. Finally, known or proposed mechanisms regarding the ecological interaction established between lactobacilli and *Candida* will be reviewed, including those potentially responsible for the yeast's inhibition.

I.2. The diversity across the human microbiome

The organisms that comprise the human microbiota colonize various anatomical sites such as the skin, the gastrointestinal tract, the respiratory tract or the urogenital tract[3, 16-23]. The characterization of the colonizing species was in the past limited by the incapacity of culturing a great number of species in the laboratory. Nonetheless with the development of culture-independent methods, for example metagenomics (e.g., 16S or ITS sequencing), our ability to identify microbial species greatly increased by removal of the culture step and simply identifying them based on the presence of their genetic material in the biological sample. Additionally, culture-dependent methods or "culturomics" have been thoroughly optimized to obtain more information regarding human-colonizing microbial species. That has been boosted by the development of more suitable culture media, coupled with improved mass spectrometry (MS) techniques that can accurately (and rapidly) identify microorganisms with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), allowing a rapid screening of large numbers of colonies[24]. Despite these advances, culture-dependent methods are still intrinsically unable to detect "non-cultivable" organisms and have a huge workload, compared with culture-independent methods. The latter has, however, the disadvantage of missing less abundant species. Thus, to have a more comprehensive view of the human microbiota, the optimal approach would be to use both culture-dependent and independent methods benefiting from a cooperative approach. Taking advantage of these approaches was possible to characterize the human microbiota to what we know currently, showing that the human host is highly populated by members of the Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria phyla (Figure I.2).

The human mouth was found to consist of hundreds of species belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, including species from the genera *Streptococcus*, *Granulicatella*, *Gemella*, *Actinomyces*, *Rothia*, *Veillonella*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Capnocytophaga*, *Neisseria*, *Eikenella*, *Leptotrichia* and *Eubacterium*[25-27]. In the gut, most likely the more often studied microbiome, there is a greater variety of species, with some being considered endogenous, while others are just transient passengers resulting from food ingestion. *Streptococcus*, *Lactobacillus*, *Prevotella*, *Clostridium*, *Veillonella*, *Staphylococcus*, *Bacteroides*, *Clostridium*, *Bifidobacterium* and *Ruminococcus* are the genera more commonly identified[28-39]. In the respiratory tract, the more commonly found species belong to *Streptococcus* genera, followed by *Corynebacterium diptheriae*, *Staphylococcus aureus*, *Haemophilus influenza*, *Mycobacterium tuberculosis*, *Bordetella pertussis*, *Klebsiella* spp., *Neisseria meningitides*, *Mycoplasma pneumonia*, *Pseudomonas aeruginosa*, *Moraxella* spp. along with *Adenovirus*, *Rhinovirus* and *Influenza* viruses[40, 41]. The most abundant colonizers of the skin are *Staphylococcus*, *Propionibacterium* and *Corynebacterium* species[17, 42, 43], however, studies have shown that the skin microbiota is hugely dependent of the skin region examined[42, 44]. For instance, *Propionibacterium* spp. dominate sebaceous areas such as the forehead, retro-auricular crease and back, whereas *Staphylococcus* and *Corynebacterium* species dominate moist areas like the axillae[42, 44]. The

most commonly isolated species from the genitourinary tract are those belonging to *Lactobacillus*, *Corynebacterium*, *Gardnerella*, *Staphylococcus* and *Prevotella* species[22, 45-50]. Interestingly, bacterial species belonging to the *Streptococcus*, *Prevotella*, *Staphylococcus*, and *Lactobacillus* genera, are found in niches that have very different characteristics (as highlighted in Figure I.2), reflecting what can be a great adaptability to colonize and thrive in the human host (e.g., *L. gasseri* is found in the vaginal and intestinal tract). It is also important to mention that identification of true endogenous species of the human microbiome is a complex subject due to interfering effects caused by the ingestion of food (e.g., in the gastrointestinal tract) or simply by direct physical contact with the environment (e.g., the skin). Thus, the scientific community have considered true colonizers of the human host species that are consistently identified in most individuals while species that are not constantly identified are considered transients organisms.

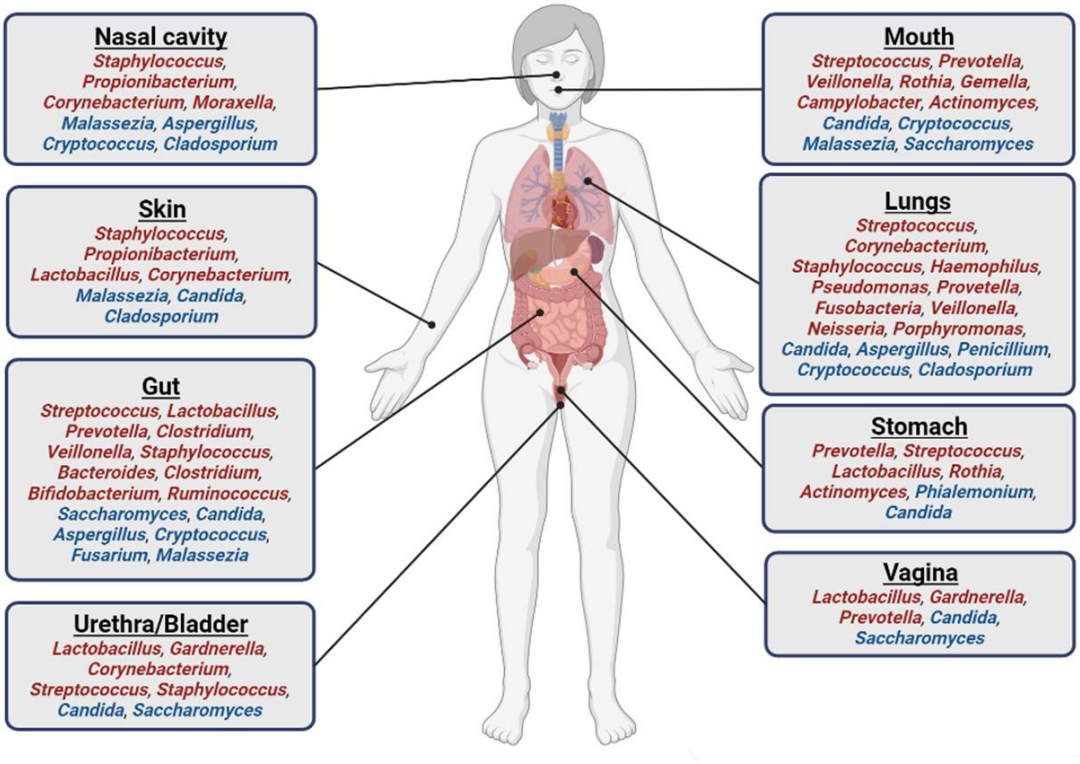


Figure I.2. Overview of the human host microbiota. Commonly found genus of bacteria (in red) and fungus (in blue) in the nose[51-55], mouth[25-27, 56-59], lungs[60-65], skin[17, 42, 43, 66-68], stomach[37, 69], gut[28-39], urethra/bladder[45-48, 70], and vagina[22, 49, 50, 71-74] of the healthy human host. Note that several genera, including *Streptococcus*, *Prevotella*, *Lactobacillus* and *Candida* are isolated from different niches. This figure was built using information to the genus level.

While the human microbiome has been fully investigated, the mycobiome, which corresponds to the fungal species that colonize the human host is substantially less studied, along with potential functional impacts on the host[74-76]. Bacterial cells vastly outnumber fungal cells, however, there are a significant number of fungal species in the gut[75], vagina[74], skin[77], mouth[59] and even in the lungs[76] (Figure I.2); with some of them only being detected with non-culture-based techniques (including amplification of ITS1, ITS2 or 18S rRNA regions[78, 79]). However, the cost of sequencing required to identify fungi in samples that are dominated by bacteria is a considerable obstacle, because it requires a higher depth of sequencing.

Nonetheless, the efforts in characterizing the human host mycobiome have determined a core of eukaryotic microorganisms in various human host niches (Figure I.2). The most usually encountered fungal species in the human host are those belonging to the *Candida*, *Cryptococcus* and *Saccharomyces* genera. In the mouth were commonly identified species belonging to *Candida*, *Cryptococcus*, *Malassezia* and *Saccharomyces*[56-59], while in the stomach only *Candida* and *Phialemonium* were detected[69]. The gut is, on the other hand, colonized by members of *Saccharomyces*, *Candida*, *Aspergillus* and *Cryptococcus*, with some reporting the presence of *Fusarium* and *Malassezia*[32-35]. However, there is a possibility that not all these species are true endogenous species of the gut since fungi are typically used in production of foods and beverages whose ingestion can cause confusion. *Malassezia*, *Aspergillus*, *Cryptococcus* and *Cladosporium*[53-55] were found in the nasal cavity, while in the lungs were encountered members from the genus *Candida*, *Aspergillus*, *Penicillium*, *Cryptococcus* and *Cladosporium*[63-65]. The skin is dominated by *Malassezia*, *Candida* and *Cladosporium*[66-68], however, this can be influenced by the direct contact with the environment since some of these species are also frequently found in the surfaces of many objects. Finally, in the genitourinary tract, were identified both in the urine and in the vagina *Candida* and *Saccharomyces* species[49, 70-74]. Like it was observed for bacteria, some fungal genera, including *Candida* and *Cryptococcus* are identified in various niches such as *Candida* that is commonly identified in vaginal, oral and intestinal samples while *Cryptococcus* are present in various oral, gut and respiratory tract samples (Figure I.2). Such ability of *Candida* spp. to colonize various human host niches is intrinsically related with their capability to adhere and invade host cells, undergo morphological transitions, form biofilms, and the presence of specific metabolic and fitness traits that confers them an advantage in the human host niches. These traits enable its survival in a variety of conditions and stresses, including low pH, temperature, other microbiota and immune system, among others, resulting in their persistence in various niches, that includes the mouth[58, 80], respiratory tract[65, 76], gastrointestinal tract[33], urogenital tract[49, 81], skin[77, 82] and even the bloodstream[83].

Despite the perceived beneficial impact of microbial homeostasis as an important contributing factor for health, several of the identified bacterial and fungal species belonging to microbiome or mycobiome of a given niche also have recognized potential to cause infection. This is, for example, the case of *Staphylococcus* species that have the potential to cause sepsis, pneumonia, osteomyelitis, endocarditis or soft tissue infections[84]; or of *Candida* spp. that are capable of causing both mucosal and systemic infections[85-89]. The ability of these fungi to shift from harmless commensals to pathogens can be linked with the immune system activity of the host and, even more importantly with the interspecies interaction between other members of the microbiome which can restrict the activity of the potential pathogens resulting in reduced pathogenicity. This aspect further increases the interest in studying the ecological interaction between species of the microbiome and mycobiome and also the interactions that these organisms establish with human cells. This intertwined interaction could be responsible for the

pathobiont behaviour of these species, contributing to a state of health or the dissemination of species able to induce disease.

I.2.1. The female vaginal microbiome

The mucosal lining of the upper genital tract (endometrium, endocervix, and Fallopian tubes) is made up of a single layer of columnar epithelial cells, while the mucosa of the lower genital tract (vagina and ectocervix) is comprised of a stratified squamous non-keratinized epithelium[90]. The mucosal surfaces are covered with a mixture of cervical mucus and vaginal transudate called cervicovaginal secretion (CVS) and are colonized by a complex cohort of microorganisms[90, 91]. Thus, epithelial cells, mucosal secretions, and microbiota are all key components responsible for maintaining vaginal health functioning as a physical, biochemical, and biological barrier against pathogens. Due to the limitations of culture-based approaches, the characterization of the vaginal microbiota in recent years has been studied using culture-independent methods. These molecular techniques, including sequencing of 16S rRNA[92, 93] and quantitative PCR[94, 95] proved crucial for the exhaustive characterization of the vaginal microbiome. Regardless of the detection methodology used, studies suggest that the healthy female vaginal microbiome is characterized by a dominance of lactobacilli species including *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus gasseri* being the most abundant[96-98]. An array of facultative and strictly anaerobic species belonging to the genera *Atopobium*, *Corynebacterium*, *Anaerococcus*, *Peptoniphilus*, *Prevotella*, *Gardnerella*, *Sneathia*, *Eggerthella*, *Mobiluncus* or *Finegoldia*, were also found in the vaginal microbiome[22, 91, 99]. Verhelst *et al.*, 2005 was one of the first studies dividing the vaginal microbial community into four types: type 1, defined as the “normal” microbiota consisting of communities dominated by *L. crispatus*, *L. jensenii*, *L. iners* and *L. gasseri*; type 2, dominated by *L. iners*, *L. gasseri*, *L. crispatus*, *Atopobium vaginae*, *Gardnerella vaginalis*, *Actinomyces neuii* and *Peptoniphilus*; type 3, characterized by the presence of BV-associated species; and type 4, characterized by a variety of *Streptococcus* species[92]. More recently, an extensive study undertaken by Ravel *et al.*, 2011, evaluated the types of bacterial communities that dominate the vaginal environment in 396 asymptomatic North American reproductive-age women representing four ethnic groups (white, black, Hispanic, and Asian). The authors distributed these microbiomes into five different community state types (CSTs), or groups (Figure I.3). These communities were dominated by *L. crispatus*, *L. gasseri*, *L. iners, L. jensenii* or formed a polymicrobial community associated with bacterial vaginosis-associated bacteria[21, 22]. Despite groups I, II, III and V were always enriched in *Lactobacillus* spp., the dominant species varied being *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, respectively[22]. In contrast, group IV was enriched in species belonging to *Prevotella*, *Megasphaera*, *Atopium* and *Sneathia* (Figure I.3), associated with BV[22].

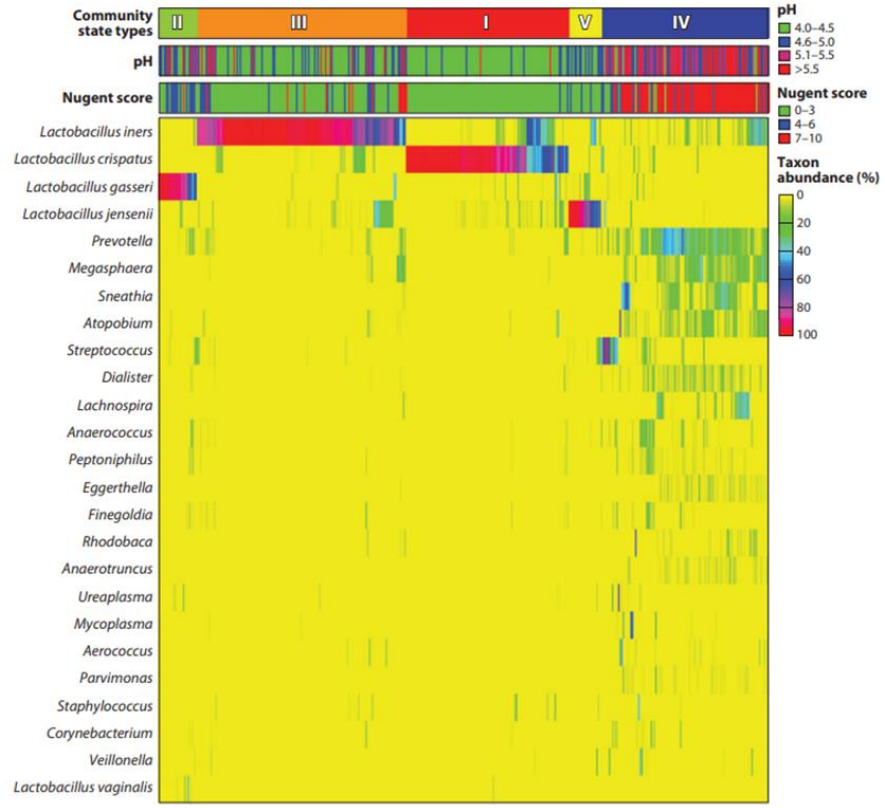


Figure I.3. Heatmap of percentage abundance of microbial taxa found in the vaginal microbial communities of 394 reproductive-age women. Samples were grouped in five different community states (CSTs) or groups. Group I, II, III and V were enriched in *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, respectively, while group IV was dominated by a polymicrobial population containing mostly *Prevotella*, *Megasphaera*, *Atopium* and *Sneathia*. Figure taken from Ravel *et al.*, 2011[22].

Similar results were obtained by Drell *et al.*, 2019 while characterizing the vaginal microbiome of 494 asymptomatic reproductive-age Estonian women, revealing a distribution of communities across 5 major groups with a clearly distinct diversity and composition, demonstrated by the principal components analysis (PCA) plot shown in (Figure I.4)[49]. The authors reported that lactobacilli were one of the most abundant members in all groups, although high predominance of members of the *Gardnerella* genus was also observed in some community groups[49]. These communities were distributed in: i) Groups I and II, highly enriched in *L. iners*; ii) groups III and IV, dominated by *L. crispatus*; iii) group V, not dominated by *Lactobacillus* but rather by a polymicrobial population enriched in *Gardnerella*, *Atopobium and Prevotella*[49], corroborating what has been previously described[22].

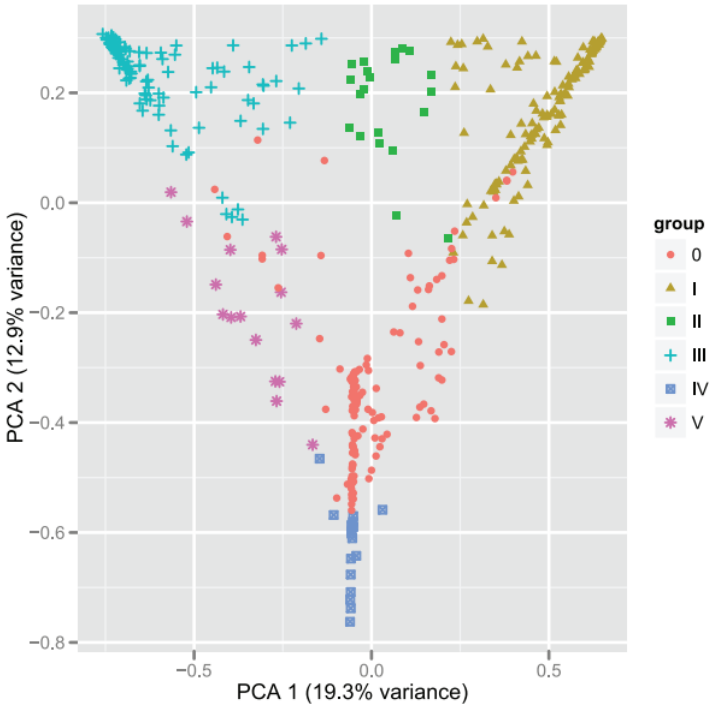


Figure I.4. PCA plot clustering the vaginal bacterial communities. Vaginal communities were clustered in five groups dominated by *L. iners* (group I and II), *L. crispatus* (groups III and IV) and a polymicrobial population (Group V). Samples clustered in group 0 were not characterized by any specific community type. Figure taken from Drell *et al.*, 2019[49].

As occurs with microbiomes from other sites, the vaginal microbiome has been much more characterized in terms of its bacterial species, while less attention has been given to the mycobiome. Nonetheless, more recent studies advanced such characterization showing that the mycobiome is dominated by Ascomycota species (such as *Candida albicans*, *Candida glabrata*, *Candida tropicalis* or *Saccharomyces cerevisiae*), with Basidiomycota and Oomycota species also being detected, but at much lower abundances[49, 81]. *Candida* species deserve, in this context, a special attention due to their well-known ability to trigger infections of the vaginal tract, as it will be further dissected in the section of this introduction that is dedicated to candidiasis. A distinguishing aspect of the pathophysiology of *Candida*, compared with other human-infecting fungi, is that these species are part of the microbiota of various niches, even in the absence of disease[75, 100, 101]. Indeed, *C. albicans* has been identified as a true gut symbiont based on its consistent identification in resident microbial populations of this niche[75, 100]. Despite *Candida* spp. have been identified in the vaginal mycobiome of “healthy” women, using both culture-dependent and independent methods[101-103], these yeasts are not ubiquitously observed in all samples suggesting that the presumed “healthy” women could be asymptomatic carriers and leaving open whether or not the vaginal tract is a primary site of colonization of *Candida* or if their identification results from a translocation of these organisms from the gut[100, 104, 105].

The vaginal environment is a complex and dynamic ecosystem continuously affected by fluctuations moved by changes in nutrient availability, pH, diet, pregnancy, hormones and exercise, for example[106-108]. The vaginal mucosa acquires oxygen, glucose, and other

nutrients from underlying submucosal tissues through diffusion, nonetheless, due to the limited blood supply this results in an anaerobic microenvironment that favours a complex microbial community that persists in a symbiotic relationship with the host[109]. In addition to the environmental factors abovementioned, choice of contraception[110], use of personal hygiene products or medications[111], the presence of sexually transmitted diseases[112], as well as numerous factors related to sexual behaviours (age at which sexual activity begins, frequency of sex, number of sexual partners, specific sexual practices engaged in, and even the introduction of semen) contribute for the regulation of the vaginal microbiota[111]. On top of that, fluctuating levels of hormones during lifetime are also crucial for the modulation of the vaginal ecosystem. Age is intrinsically associated with the estrogen concentration in the vagina which is significantly lower in the premenarchal and menopause compared with the reproductive years[113]. Before the first menstruation (premenarcheal), young women were found to be highly colonized by diphtheroids, *Staphylococcus epidermidis*, α-hemolytic streptococci and *Escherichia coli*[114-117]. Interestingly, lactobacilli were isolated most frequently from older girls while enteric organisms were isolated most frequently from younger girls, suggesting that lactobacilli colonization could be age associated[114-117]. During puberty, approximately 70% of young women exhibited the presence of *Lactobacillus* spp., with sexually active adolescents having nearly double the likelihood of carrying a higher concentration of lactobacilli compared to their non-sexually active counter-partners[118]. During reproductive age, women have high amounts of estrogen and their vagina is colonized, as stated, mostly by lactobacilli. In the menopause, women suffer a reduction in estrogen production, resulting in drying and atrophy of the vaginal epithelium[119]. Reduction in estrogen greatly affects the vaginal environment resulting in the depletion of lactobacilli and subsequent rise in vaginal pH because of the reduced production of lactic acid[120]. These changes promote the growth of pathogenic bacteria, particularly colonization by enteric species[113]. In fact, it was reported that only 46.3% of postmenopausal women had *Lactobacillus*-dominated populations[121]. Although nearly half of the postmenopausal women retained colonization with lactobacilli, it was observed that their concentrations were 10 to 100 times lower than those observed in women of reproductive age[121, 122]. Interestingly, when postmenopausal women were subjected to hormonal treatment, such as the administration of estrogen, a reduction in the vaginal pH was observed, concomitant with an increase of the lactobacilli abundance, reinforcing the important role of estrogen in shaping the vaginal microbiota[119, 120, 122]. Such role of hormones in modulation of the vaginal microbiota was also reported in association with the use of hormonal contraceptives. In specific, the use of estrogen-containing contraceptives resulted in a decreased risk of bacterial vaginosis (BV) recurrence and increased concentrations of *Lactobacillus* in the vagina[123, 124], corroborating the previous evidence that estrogen favours a vaginal microbiome dominated by *Lactobacillus*[21, 125, 126]. Another factor that could influence the vaginal microbiota is menstruation. Although it is generally accepted that it has a strong influence on the stability of the vaginal ecosystem, reports have shown conflicting results. Some studies have reported that during menstruation *Lactobacillus* spp. tended to decrease in abundance and *Streptococcus* spp.,

Peptostreptococcus spp., and *Anaerococcus* spp. tended to increase, suggesting that microbiome diversity increases during menses, concurrent with a decrease in *Lactobacillus* abundances at the genus level[106]. Differently, other studies show that the vaginal microbiome of most women remained relatively stable throughout the menstrual cycle, typically dominated by *L. crispatus*, *L. iners*, and *L. jensenii* with little variation in diversity and only modest fluctuations in species richness[127].

Vaginal dysbiosis results from the disruption of the usual microbial communities established in the vagina and is, if not treated, associated with several gynaecological diseases, such as pregnancy loss, preterm labour, and low conception rates[128, 129]. Vaginal dysbiosis is usually accompanied by a change in the vaginal pH, caused by a reduction of the lactic acid produced by lactobacilli species[130]. Indeed, vaginal health has been well linked with a lactobacilli-enriched microflora (predominantly in *L. crispatus*, *L. gasseri L. jensenii*, or *L. iners*[22, 49, 98]) that is associated with absence of vaginal symptoms, lower risk of infections and good pregnancy outcomes[92, 130, 131] (Table I.1). Thus, dominance by *Lactobacillus* spp. of the vaginal microbiome is a useful biomarker for evaluating health and disease.

The beneficial role of the lactobacilli-enriched microflora has been particularly well established for bacterial vaginosis (BV) (Table I.1). BV is a common vaginal dysbiosis among women of reproductive age, characterized by the overgrowth of opportunistic bacteria (e.g., *Prevotella* spp. and *G. vaginalis*) and decreased abundance of lactobacilli[132, 133]. Verhelst *et al.*, 2005 was one of the first studies dividing the vaginal microbial community and correlating the lower abundance of *Lactobacillus* spp. with the presence of BV-associated species[92]. Later on, other studies substantiated these findings by linking the decrease in vaginal lactobacilli (in particular *L. crispatus*) with the increased prevalence of BV-causative species such as *A. vaginae*, *Leptotrichia amnionii*, *Prevotella amii* or *Fusobacterium gonidiaformans*[22, 93, 131]. Notably, these studies observed that *L. iners* was abundant in all women, including those who suffered from BV, while *L. crispatus* was almost exclusively isolated from healthy women[93]. This finding suggested that within *Lactobacillus*, not all species could contribute equally for health maintenance in the vaginal environment, as schematically represented in (Figure I.5).

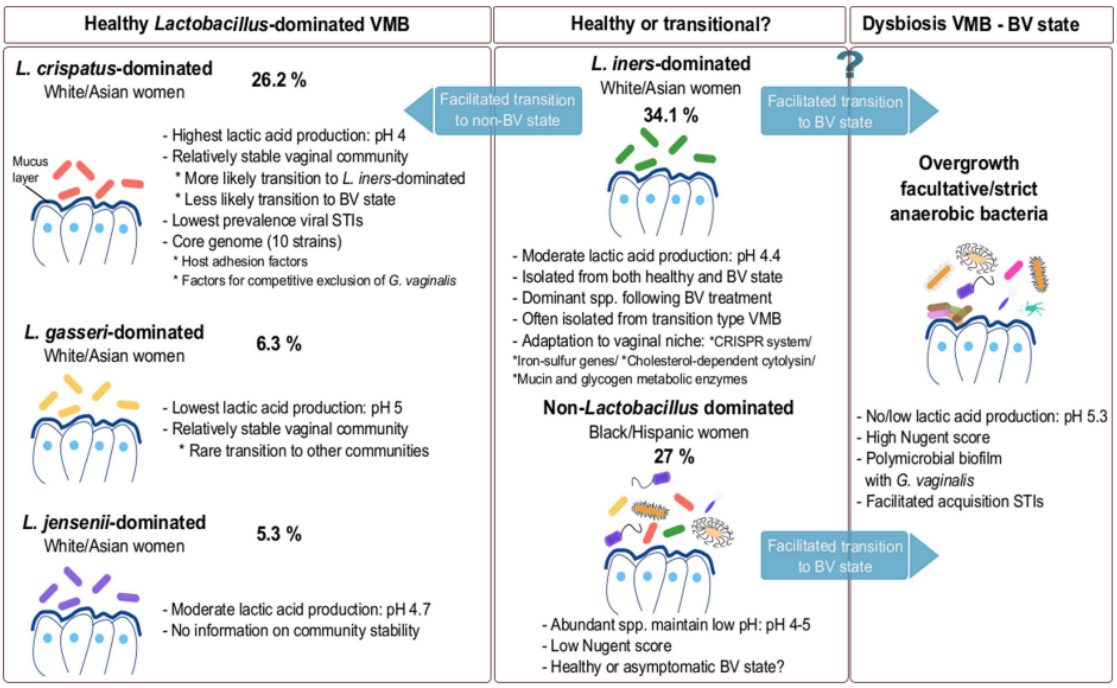


Figure I.5. Model proposed by Petrova *et al.*, 2015 for the composition of the vaginal microbiome during healthy and their transition to a dysbiosis state. Healthy women vaginal communities can be divided into different groups. Although the exact number of communities and their composition is still under study, most reports on this subject observed that *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* are predominant in healthy subjects while *L. iners* was associated with a transition from healthy to disease condition. Moreover, some of these studies identified a non-*Lactobacillus*-dominated VMB in various healthy individuals[21, 22, 92, 110, 134]. Figure taken from Petrova *et al.*, 2015[134].

Table I.1. Example of studies associating the role of *Lactobacillus* spp. abundance with the development of vaginal infections. The table describes the samples, the designed methodologies, the disease or disease causing pathogen and the major findings of the study. Overall, the presence of *Lactobacillus* was found to be crucial for the health status of the vagina, suggested by differences in the bacterial communities of healthy (dominated by *Lactobacillus*) and diseased women. Abbreviations: BV – bacterial vaginosis; VVC – vulvovaginal candidiasis; CTI – *C. trachomatis* infection.

Study	Samples	Design/Microbiota determination	Pathogen/infection	Major findings
Rita Verhelst *et al.*[92]	515 vaginal swabs from 197 pregnant women	Vaginal swabbed samples were smeared onto a glass slide and gram stained for bacterial identification. *G. vaginalis* and *A. vaginae* were further quantified using 16S rRNA amplification	*G. vaginalis* and *A. vaginae*	Bacterial communities characterized by the absence of *L. crispatus* have a higher risk of developing BV. *A. vaginae* abundance was associated with the development of VVC
Ruben Hummelen *et al.*[131]	132 vaginal swabs from Tanzanian women with HIV, including 39 diagnosed with BV and treated with metronidazole	Bacterial load of the vaginal samples was determined using PCR amplification of the V6 sequence of the rRNA	*G. vaginalis*, *Prevotella bivia*, *Lachnospiraceae*, multi-species	The presence of *L. crispatus* was associated with a healthy pH<4.5, and a reduced risk of developing BV
Sujatha Srinivasan *et al.*[93]	220 vaginal swabs from women with (98) and without BV (122) (based on Amsel's criteria)	Bacterial load of the vaginal swabs of women with and with BV was compared and determined using pyrosequencing of 16S rRNA	*Megasphaera* spp., *L. amnionii*, *Sneathia sanguinegens*, *G. vaginalis* and *A. vaginae*	The presence of healthy associated *L. crispatus* was inversely correlated with the abundance of BV associated bacteria
Camilla Ceccarani *et al.*[130]	Vaginal swabs of healthy (21), BV (20), CTI (20) or VVC (18) diagnosed women	Microbiome of women diagnosed with BV, CT or VVC were compared with healthy women. Bacterial load was determined using 16S rRNA gene sequencing	Bacterial vaginosis (BV), *Chlamidia trachomatis* (CTI), and vulvovaginal candidiasis (VVC)	Vaginal health was characterized by an abundance of lactobacilli. Development of CTI, VVC, and BV was accompanied by a reduction of *L. crispatus* and a progressive replacement by *L. iners*
Carola Parolin *et al.*[135]	Vaginal swabs of healthy (22), BV (19) and CTI (20) diagnosed women	Vaginal bacterial communities were determined using VaginArray, a phylogenetic DNA microarray able to detect the most representative species of the human vaginal microbiota in eubisosis and dysbiosis	*C. trachomatis* (CTI)	CT infection was characterized by bacterial and metabolic signatures similar to a healthy condition, even though higher amounts of *L. iners* were observed. The abundance of *L. crispatus* was higher in asymptomatic CTI-positive patients than in women with CTI-correlated symptoms.

The protective effect of lactobacilli against other vaginal pathogens is far less clear. For example, in the case of vaginal candidiasis conflicting results had been published with some studies reporting no significant difference in the abundance of vaginal lactobacilli in women diagnosed with this infection[102, 136-138] while in others reduced densities of the *Lactobacillus* populations are reported[139]. As also suggested for bacterial infections, it has been shown that women suffering from *Candida* infections may have an overall similar abundance of lactobacilli compared with healthy individuals, but the predominant species is *L. iners*[130] and not *L. crispatus* or *L. gasseri*[101, 140]. Compared to these two species, *L. iners* was recently reported to lack a cysteine biosynthetic pathway and also a restricted repertoire of mechanisms for the uptake of this amino acid which can prompt a lower competitiveness over vaginal pathogens[141].

I.2.2. The intestinal microbiome

The gastrointestinal tract (GIT) microbiome is a dynamic and functional interface between the external environment, the ingested food, and the human host. It changes spatially and temporally in relation to an individual health condition. The microbes that colonize the GIT can shift in composition as humans age, reaching the highest diversity during adolescence and adulthood[142]. During childhood and adolescence, the GIT microbiome is mostly composed of *Bifidobacterium* spp., *Faecalibacterium* spp., and species of the *Lachnospiraceae* family[142]. As humans age, microbial diversity increases steadily in healthy individuals[143, 144] to encompass species belonging to *Bacteroides*, *Faecalibacterium* and *Bifidobacterium*, together with aerobes or facultative anaerobes belonging to *Enterobacter*, *Klebsiella*, *Enterococcus*, *Lactobacillus*, *Escherichia*, and *Proteus*, usually less identified (Figure I.6)[16, 145, 146]. On top of the age factor, the microbial composition of the GIT also varies according to the anatomic site. Upstream jejunal samples showed an abundance of *Streptococcus*, *Prevotella*, *Veillonela*, *Fusobacterium*, *Escherichia*, *Klebsiella*, and *Citrobacter*[147] and as the small intestine progresses distally in the ileum, the microbial composition becomes more complex and starts to resemble those found in the colon concerning diversity and richness[148]. The colon is dominated by Bacteroidetes, Firmicutes, Verrucomicrobia, Proteobacteria, and Actinobacteria[39].

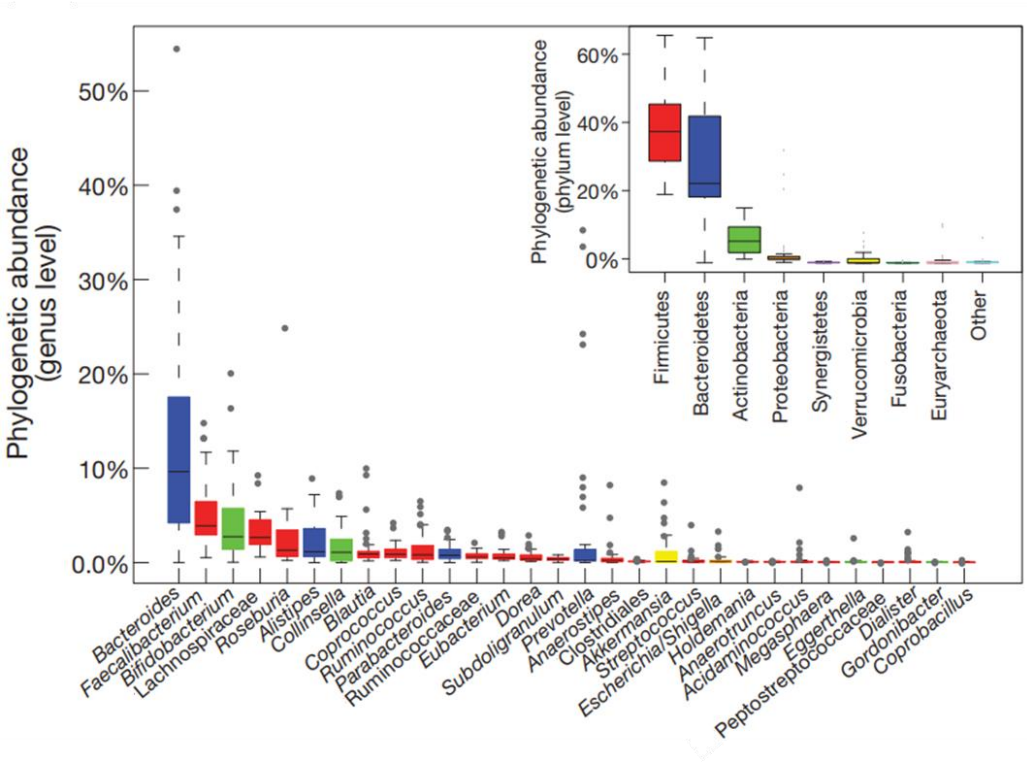


Figure I.6. Predominant genera determined by metagenomic analysis of the human gut belonging to 39 faecal samples from six nationalities revealed that the gut is characterized by a great biodiversity while being dominated by the phyla Firmicutes and Bacteroidetes, and the genera *Bacteroides*, *Faecalibacterium*, and *Bifidobacterium*. Figure taken from Manimozhiyan Arumugam *et al.*, 2011[16].

As denoted before, the distinction between true GUT symbionts and transient species (originating from the ingestion of foods) complicates the task of identifying a "core" gut microbiome[100, 149]. This is relevant in the case of lactic acid bacteria since many of these species are explored by the food industry to produce fermented foods. Despite this, efforts have been made to distinguish between endogenous and transient species where among the many *Lactobacillus* species found in the gut, *L. gasseri*, *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), *Ligilactobacillus ruminis* (formerly *Lactobacillus ruminis)* and *Ligilactobacillus salivarius* (formerly *Lactobacillus salivarius*) were considered true endogenous species due to their constant presence and the long-term colonization of this niche[150]. Besides these abovementioned species that are believed to be true gut symbionts, metagenomic analyses have also unveiled the presence of many other lactobacilli species, albeit in a more inconsistent manner among samples. Examples of these species are *Lactobacillus acidophilus*, *Levilactobacillus brevis* (formerly *Lactobacillus brevis)*, *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *L. crispatus*, *Lactobacillus delbrueckii*, *Limosilactobacillus fermentum* (formerly *Lactobacillus fermentum), L. jensenii*, *Lactobacillus johnsonii*, *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) and *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*)[150-156]. Notably, some of these lactobacilli species are also found in the oral cavity (such as *L. plantarum*, *L. salivarius* and *L. acidophilus*[157]) suggesting a possible translocation of these species across the gastrointestinal tract.

The GIT microbiome is also populated by various fungal species. Nash *et al.*, 2017 studied the faecal mycobiome of 317 healthy donors from the Human Microbiome Project reporting high prevalence of species belonging to the *Saccharomyces*, *Malassezia* and *Candida* genera, with the highest abundance attributed to *S. cerevisiae*, *Malassezia restricta* and *C. albicans*, respectively[33]. A previous study examining 98 subjects have also found many fungal taxa in common in the samples, with the top three being *Saccharomyces*, *Candida*, and *Cladosporium*[158]. Similarly, to the microbiome, the identification of the true intestinal mycobiome is complex and to overcome this challenge, Fiers *et al.*, 2019 defined a set of "rules" with the purpose of identifying true fungal symbionts of the human gut, these being: 1) detection by both sequencing and culturomics; 2) activation of host immune responses; 3) ability to populate and thrive in the gastrointestinal niche; and 4) evidence of trans-kingdom interactions between bacteria and fungi. Using these "rules", the authors determined that among *Candida* spp. only *C. albicans* could be accepted as a true symbiont of the human gut[100]. In addition to *C. albicans*, other *Candida* spp. like *C. glabrata* and *C. tropicalis* are also commonly identified in human gut samples[33, 101-103].

The gut microbiome, like the vaginal microbiome, is subject to a wide range of both internal and external influences that modulate its composition (Figure I.7)[145, 159]. These factors include the method of delivery during childbirth[160], the genetic makeup of the host[161], the immune response of the host[162], dietary habits[7], exposure to xenobiotics (in particular antibiotics)[163], infections[164], diurnal rhythm[165], exposure to microorganisms in the environment[166] and age[167]. The first year of life represents a significant period of fluctuation and maturation of the GIT microbiome. In this period there is a relatively low taxonomic diversity right after birth, but over time it increases as the infant is colonized with bacteria acquired from breast milk and the environment[168, 169]. Diet is a significant driver for the development of the infant microbiome as it adapts to the changing availability of nutrients. Early in infancy, the GIT microbiome is enriched in genes involved in the digestion of oligosaccharides found in breast milk, while later in infancy, due to the introduction of solid foods, the microbiome is enriched in genes involved in the digestion of polysaccharides[160, 170, 171]. In addition to infant diet, environmental and pharmacological exposures are also associated with differences in the developing infant microbiome including antibiotic exposure[172, 173], number of siblings[172], exposure to pets[174], day care attendance[169] and geography[175]. During childhood, the gut microbiota of American children had reported to have an increased abundance of Firmicutes[142, 176], Proteobacteria[177] and Actinobacteria[142, 176], while abundance of Bacteroidetes decreases[177], compared to the microbiome of adult population. Differences in the microbiome of adults and children had also been observed at a more functional level, with the GIT microbiomes of children being enriched in functions supporting ongoing development such as biosynthesis of vitamin B12[142]. The production of sexual hormones, starting in the adolescence, was also found to modulate the gut microbiota with the levels of testosterone and estradiol levels in men and women being associated with the abundance of *Acinetobacter*, *Dorea*, *Ruminococcus*, and *Megamonas* in the gut of men, and more abundance of Bacteroidetes and less Firmicutes

phyla in women gut[178]. During adulthood the gut microbiome remains relatively stable, mostly dominated by Firmicutes, Bacteroidetes and Proteobacteria[146], except for perturbations induced by infections, antibiotic treatment or drastic dietary interventions[7]. Even though the GIT microbiome recovers to its initial state relatively quickly after these perturbations[7, 179] they can alter microbial composition over time and are likely strong drivers behind the extensive strain-level interindividual differences observed among healthy adults[180]. Finally, in the elderly there is a higher Firmicutes to Bacteroidetes ratio when compared to adults[181] with a concomitant reduction in protective commensal *Bifidobacteria* and *Bacteroides*[181] that is believed to result in an overall decrease in life quality[182].

On top of age, the gut microbiome can also be modulated by other factors. Geographical location affects the gut microbiome to a great extent, possibly attributable to variations in dietary habits or other environmental influences[183, 184]. For example, elderly individuals that live in a community setting have higher proportions of Firmicutes to Bacteroidetes, in comparison to long-stay residential individuals[185]. Data from the Human Microbiome Project has also found that the human microbiome fingerprint is very similar between individuals that share the same home[186]. Host genetics also influences the gut microbiota, where heritability of *Christensenellaceae* in the gut did not appear to be driven by diet but possibly due to host genetic factors[161]. Gender-specific differences in GIT microbiome composition have also been observed, which may be explained by combinations of host genetics and social context[23, 187]. Dietary is another important factor able to modulate the gut. Fibre-rich diets can have short term-impacts on the GIT microbiome[7], but more importantly, long-term fibre intake has been linked to microbiome beneficial properties[188]. For example, the intake of fibre-rich diets promotes the colonization of *Prevotella* spp. and *Xylanibacter* spp., two genera capable of degrading nondigestible fibres. Children from Burkina Faso, which have a diet rich in fibre have a higher abundance of these species compared to European children[189], whose diet includes more processed foods, simple carbohydrates, animal protein, fat and is low in fibres[189]. The administration of antibiotics leads to decreased bacterial diversity and richness, with outcomes ranging from mild diarrhoea to life-threatening pseudomembranous colitis[190]. Although the GIT microbiome is restored after approximately one month without antibiotic administration, some taxa are permanently lost[191, 192]. The abovementioned studies are only a small portion of the enormous number of studies focused on factors that could influence the gut microbiota. Altogether, these evidences demonstrate that the human gut is highly influenced simultaneously by microbiome intrinsic, environmental, host intrinsic and host extrinsic factors that contribute for the differences observed between host microbiomes (Figure I.7). The study of how these factors impact the gut microbiota is crucial for the understanding of microbiome associated diseases, including infection caused by pathogens.

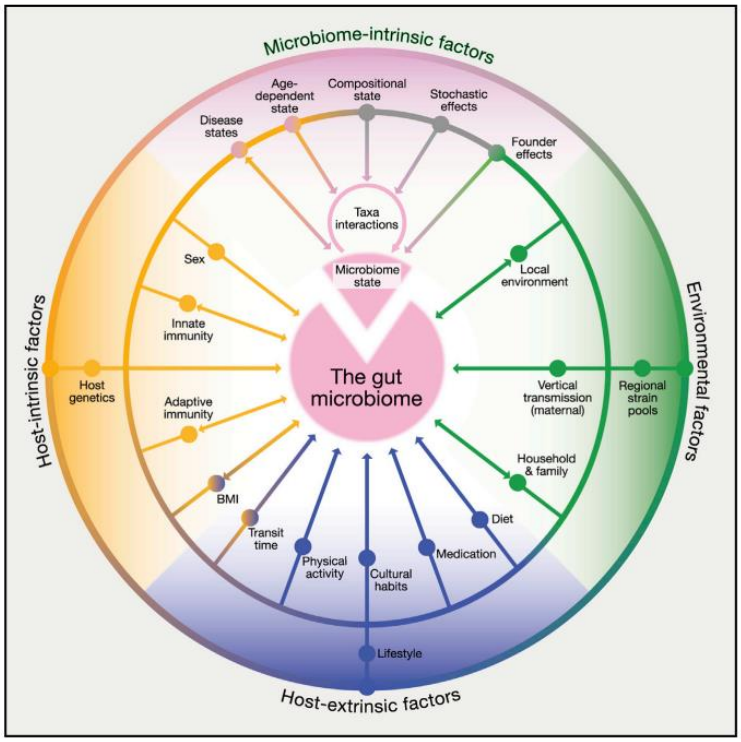


Figure I.7. Human gut microbiota is modulated by several microbiome intrinsic, environmental, host intrinsic and host extrinsic factors including diet, age, medication, geographical location, sex and immune system, among many others. Together these factors contribute to the establishment of a healthy gut microbiota or under certain conditions, for example, the administration of antibiotics or ageing, for a dysbiosis associated with negative effects for the host. Figure taken from Thomas S.B. Schmidt *et al.*, 2018[193].

There is growing evidence that dysbiosis of the gut microbiota is associated with the development of diseases, including inflammatory bowel disease (IBD)[194], coeliac disease[195, 196], allergy[197], cardiovascular disease[198], obesity[199] and infections[200-204]. Gut microbiota dysbiosis was also associated with various infectious diseases including infections caused by *Clostridium difficile*, *Helicobacter pylori*, Hepatitis C virus or bloodstream infections (Table I.2). *C. difficile* causes disease that ranges from mild diarrhoea to severe pseudomembranous colitis[205] and stool samples gathered from patients infected with this bacterium were characterized by a depletion of Bacteroidetes as well as of several species described as antagonists against *C. difficile*[200]. An extensive study performed on the Chinese population also revealed that *H. pylori*-positive group had a higher abundance of *Prevotella*, whereas the healthy *H. pylori*-negative group had a higher abundance of *Bacteroides* and that vitamin B12 biosynthesis module was depleted in the *H. pylori*-positive group[201]. Other examples of the association between gut microbiome dysbiosis and diseases include the reduced bacterial diversity and abundance of *Clostridiales* and increase of *Streptococcus* and *Lactobacillus* in patients infected with Hepatitis C virus[202] and the development of secondary bloodstream infections in COVID-19 patients[203].

Table I.2. Example of studies associating the role of intestinal microbiota with the development of intestinal infections. Table describes the samples, the designed methodologies, the disease or disease causing pathogen and the major findings of the study. Overall, under intestinal infected conditions, the host gut microbiota is significantly altered compared with the healthy gut microbiota. Abbreviations: CDI - *C. difficile* infection; HPI - *H. pylori* infection; CHC - chronic hepatitis C virus infection; HCV - Hepatitis C virus infection; IBD - inflammatory bowel disease; DEC - Diarrheagenic *Escherichia coli*

Study	Samples	Design/Microbiota determination	Pathogen/ infection	Major findings
Sophie Amrane *et al.*[200]	11 stool samples from patients with CDI and 8 stool samples from healthy patients	Culturomics and metagenomics analysis of the gut microbiota composition between CDI and controls	*C. difficile*	Significant depletion of Bacteroidetes in the CDI group (5.3%) compared to the control group (18.2%)
Daoming Wang *et al.*[201]	128 stool samples from patients with HPI and 185 stool samples from healthy patients	Metagenomic analysis of stool samples from HPI and healthy subjects	*H. pylori*	Gut microbial and plasma vitamin B12 biosynthesis were significantly lower in *H. pylori*-positive individuals; *H. pylori*-positive group had a higher proportion of *Prevotella*, whereas the healthy *H. pylori*-negative group had a higher abundance of *Bacteroides*
Takako Inoue *et al.*[202]	166 stool samples from CHC patients and 23 stool samples from healthy subjects	Gut microbiota community was analysed using 16S ribosomal RNA gene sequencing	Hepatitis C virus	Bacterial diversity was lower in persons with HCV infection, with a decrease in the order *Clostridiales* and an increase in *Streptococcus* and *Lactobacillus*
Mericien Venzon *et al.*[203]	130 stool samples from SARS-CoV-2 infected patients	Metagenomic analysis of stool samples from patients infected with SARS-CoV-2	Secondary bloodstream infections (e.g., caused by *Staphylococcus*)	Secondary bloodstream infection in COVID-19 patients was associated with gut microbiome dysbiosis, being negatively associated with the presence of the immunosupportive *Faecalibacterium*
Harry Sokol *et al.*[204]	Stool samples from 56 IBD patients, including 8 infected with *C. difficile*, 24 without *C. difficile*, and 24 patients in remission, as well as 24 healthy subjects	Comparison of 16S rRNA gene sequencing of stool samples from IBD patients infected or not with *C. difficile* with stool samples from healthy subjects	*C. difficile*/ inflammatory bowel disease (IBD)	Reduced abundance of *Blautia* and *Dorea* in CDI patients and increased abundance of *Ruminococcus gnavus* and *Enterococcus* in IBD-only and CDI patients.
Pablo Gallardo *et al.*[206]	63 diarrheal stool samples from children	Bacterial load was determined using FilmArray® GI which is an FDA-cleared qualitative PCR system that detects gastrointestinal pathogens	Diarrheagenic *E. coli*	Higher abundance of Bacteroidetes and Proteobacteria in the DEC group compared to the healthy group. DEC group was characterized by low numbers of Firmicutes compared with the healthy children

Even though *Lactobacillus* spp. are recurrently identified from human intestinal samples[39, 207], they have been shown to represent only between 1% to 6% of the total bacterial community found in the GI tract[207, 208]. Nonetheless, members of this genus have a long history of being associated with good intestinal health. This notion comes from studies showing the benefit of probiotics administration, nonetheless the importance of resident intestinal *Lactobacillus* populations in prompting health remains elusive. Studies suggest that several diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), rheumatoid arthritis (RA), type 1 and type 2 diabetes, multiple sclerosis (MS), obesity, cancer and cognitive development and behaviour were all linked with the abundance of lactobacilli in the gut, however, while in some pathologies is observed a decrease of these populations (e.g. in irritable bowel syndrome (IBS)[209], multiple sclerosis (MS)[210] or in colon cancer[211]), in others (e.g. Crohn's disease[212], rheumatoid arthritis[213], breast and head and neck squamous cell cancer[214, 215]) they appear to increase.

I.3. *Lactobacillus gasseri* and *Limosilactobacillus reuteri* (former *Lactobacillus reuteri*) - a brief overview of physiology

L. gasseri and *L. reuteri* are two endogenous lactobacilli species found in the human host. While *L. gasseri* is isolated from both intestinal and vaginal environments, *L. reuteri* is almost exclusively identified in the gut. In this thesis these species are explored and because of that the following sections intend to provide an overview about relevant aspects of their physiology and biology.

As described above *L. gasseri* is one of the most abundant species found in the vaginal tract. This Gram-positive bacterium consists of rod-like cells that can be organized in single or chain morphology, being non-mobile and non-flagellated. Growth of *L. gasseri* is greatly enhanced by anaerobiosis and in environments with a low concentration of CO_2. It can utilize glucose, fructose, sucrose, cellobiose, and salicin[216], almost always using a homofermentative carbon metabolism that leads to the production of lactic acid. Their optimal growth temperature ranges from 35 to 38 degrees, with no growth detected below 22 degrees[216]. *L. gasseri* can successfully sustain low pH environments (about 2) and, besides the vaginal and the intestinal tract, it can also be found in the mouth[216]. The *L. gasseri* reference strain ATCC 33323, isolated from stool, has a genome with around 1.89Mb in size (GC content ~35.3%; Bioproject - PRJNA84) that encodes approximately 1755 proteins. Besides chromosomic DNA, some strains also harbour a plasmid that is known for the coding of gassericin, a bacteriocin[217]. This small peptide known for its antimicrobial properties is not the only one encoded by *L. gasseri*, helveticin J, encoded in the chromosomal DNA is also frequent in most strain (including in the reference strain ATCC 33323) as well acidocin[218, 219]. *L. gasseri* cells were also reported to produce significant amounts of H_2O_2 (of about 0.8mM)[220] as well as biosurfactants[221], although the exact chemical nature of these molecules is not characterized yet. *L. gasseri* ATCC 33323 also encodes

14 mucus-binding proteins (MUBs), crucial for host adhesion, six of which exhibit a signal peptide and four predicted to be covalently linked to the membrane via sortase A cleavage of the LPTXG motif[222]. Additionally, it also been documented that this species displays the production of exopolysaccharides (EPS)[223], consistent with the identification of at least one EPS biosynthesis gene cluster. This noteworthy aspect will be discussed in further detail in a dedicated section of this thesis.

L. reuteri is commonly isolated from human faeces and has a slightly irregular morphology, bent Gram-positive rods with rounded ends and appears to associate in pairs or in small clusters[224]. Just like *L. gasseri*, it is also non-motile and non-flagellated with optimal growth in low oxygen conditions or anaerobiosis at 35 to 38 degrees[224]. It can tolerate acidic environments (with a pH~5) but also more alkaline ones (up to pH 7.5) with the optimal being between 6 to 6.8[224], which could explain with it is frequently isolated from the gut and not the vagina like *L. gasseri*. *L. reuteri* is able to consume glucose, fructose, arabinose, ribose, sucrose, lactose, maltose, melibiose, raffinose and gluconate, while producing CO_2, acetic acid or ethanol and both isomers of lactic acid (D and L), thus having a heterofermentative metabolism[224]. *L. reuteri* ATCC 23272 reference strain contains a genome of around 1.99Mb encoding approximately 1900 proteins with around 38.9% GC content (Bioproject PRJNA15766 at NCBI) with some strains able to produce the antimicrobial compounds reuterin (3-hydroxypropionaldehyde (3-HPA))[225, 226] and reutericyclin[227, 228] as well as the bacteriocin reutericin[229, 230]. *L. reuteri* is able to produce reuterin by converting glycerol into this compound using a coenzyme B12-dependent glycerol dehydratase (GDH) under anaerobic conditions. This dimeric enzyme contains three subunits (α2, β2, and γ2) encoded by the genes *pduCDE* in the *pdu* operon and is responsible for biosynthesis of 3-HPA[231, 232]. *L. reuteri* was also reported to produce considerable amounts of biosurfactant[233, 234], H_2O_2[235, 236] and to stimulate the immune system[237-239], all these being potential factors contributing to its probiotic properties. *L. reuteri* is especially well suited for the colonization of the gut, mostly because of its ability to attach to mucin and the intestinal epithelia through expression of mucus-binding proteins (MUBs) and MUB-like proteins[240, 241] capable of efficiently adhering to mucus. The considerable diversity of MUBs among *L. reuteri* strains and the variation in the abundance of cell-surface MUBs significantly correlates with their mucus-binding ability, suggesting an evolution that improved its ability to bind intestinal cells and colonize the human gut[240]. The presence of a biosynthesis EPS gene cluster in *L. reuteri* strains was also reported[242].

Evidences of the physiological and molecular mechanisms prompted by *L. gasseri* and *L. reuteri* to colonize the human host are still only few, reflecting what is a still poor knowledge about the biology and physiology of these species, especially compared to other lactobacilli that are far more studied. The vaginal environment is dominated by hydrogen producing bacteria (including *L. gasseri* itself) and thus it is expected that this bacterium must have evolved adaptative mechanisms rendering it able to cope with H_2O_2 in the environment. Although there is no study directly evaluating the effects of oxidative stress over *L. gasseri* cells, the genome of this species includes a thioredoxin (*LGAS_0427*; TrxA) and thioredoxin reductase (*LGAS_1325*; TrxB) which

could have a role in oxidative stress response in this species. *L. gasseri* is also equipped with a set of ClpATPases (ClpE, ClpC, ClpL, and ClpX) in the dependence of the HrcA operon, that were involved in the response to heat stress in this species[243]. Additionally, the predicted chaperone ClpL (*lr1516*), a putative esterase (*lr1516*) and a putative phosphatidylglycerophosphatase (*lr1797*) were required for *L. reuteri* survival at low pH[244]. BlastP analysis also revealed that *L. gasseri* encodes a set of transporters (WP_003657106.1, WP_003656709.1, WP_003647559.1 and WP_003646748.1), belonging both to the Major facilitator (MF) or the ABC Superfamilies, homologous to those described to actively extrude bile salts in *L. acidophilus*[245]. Future studies will be required to determine whether these proteins will have a similar function and contribute for the capability of this species to thrive in the intestinal tract. Three homologues of these MFS transporters were also identified in the genome of *L. reuteri* (WP_267493717.1, WP_168240781.1 and WP_019253162.1). In *L. reuteri* bile tolerance was also found to be significantly reduced by deletion of the Clp chaperone (*lr1864*), by the putative esterase *lr1516* and by the multidrug resistance transporter *lr1584* (homolog of WP_019253162.1)[246]. Genome analysis has also predicted that both *L. gasseri* and *L. reuteri* species encode at least a house-keeping sigma factor (rpoD, *LGAS_RS05465* and *C6H63_05440*) while *L. gasseri* encodes two σ^{70} sigma factors (*LGAS_RS01695* and *LGAS_RS07205*)[222] and *L. reuteri* encodes only one σ^{70} sigma factor (*C6H63_05025*) (data obtained from NCBI).

I.4. The relevance of *Candida* species as human pathogens

The genus *Candida* includes approximately 160 species, some of which are adapted to live in mammalian hosts, causing infections in both immunocompetent and immunocompromised individuals[247]. *C. albicans* is the most commonly isolated *Candida* species from faecal samples, however, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. tropicalis* are also frequently identified[248]. In the vagina, more than 70% of the *Candida* strains isolated belong to *C. albicans*, nonetheless, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* have also been isolated from asymptomatic women[49, 101, 249]. Both systemic and vulvovaginal candidiasis are more commonly caused by *C. albicans*[250-253], however, the number of infections attributable to non-albicans *Candida* species (NACS) is on the rise, particularly those caused by *C. glabrata* or *C. parapsilosis*[254-256]. Infections caused by NACS are worrisome since these species are usually more tolerant than *C. albicans* to commonly used antifungals and, thus, are associated to poorer outcomes for patients[257, 258]. Among NACS, *C. glabrata* is usually the more prevalent species, in part due to its innately higher tolerance to azoles (specially to fluconazole) and extreme genomic plasticity that, among other traits, prompts fast adaptive responses to the challenging environment of infection sites[257, 259].

Infections caused by *Candida* are mainly superficial, but in severely immunocompromised or hospitalized patients (especially those using invasive indwelling medical devices) systemic infections (known as candidemia) can occur[260, 261]. Candidemia accounts for 9% of all nosocomial bloodstream infections[262, 263] and is associated with prolonged

hospital stays and increased healthcare costs[264]. Candidemia mortality rate ranges from approximately 20% to 75% of all diagnosed cases, depending on the causative etiological agent and also of the patient conditions[265-267]. While candidemia is linked with specific risk factors (out of which the impairment of the immune system is the more important one), superficial candidiasis is very common even among the “healthy” population. Most women are colonized by *Candida* species at some point in their lives, resulting in asymptomatic colonization or in a symptomatic infection known as vulvovaginal candidiasis. Historically the development of candidemia was associated with the use of indwelling medical catheters that could be colonized by *Candida* resulting in the direct introduction of the yeast in the patient’s bloodstream. However, more recently it has grown the idea that the resident *Candida* populations present in the gut can also serve as reservoirs for dissemination, invading the epithelial layer and propagating into the bloodstream. Such behaviour was well described to occur for *C. albicans*[268]. In “healthy” conditions, *C. albicans* colonization of the gut is asymptomatic, however, under certain conditions, due to hyphal penetration or rupture in the epithelial layer *C. albicans* cells can disseminate into the bloodstream and colonize major organs (Figure I.8)[268, 269]. It has also been suggested that the vaginal tract may serve as driveway for dissemination of *Candida*[270, 271], supported by the observations that blood isolates of septic patients were similar to the ones obtained from genital swabs[272].

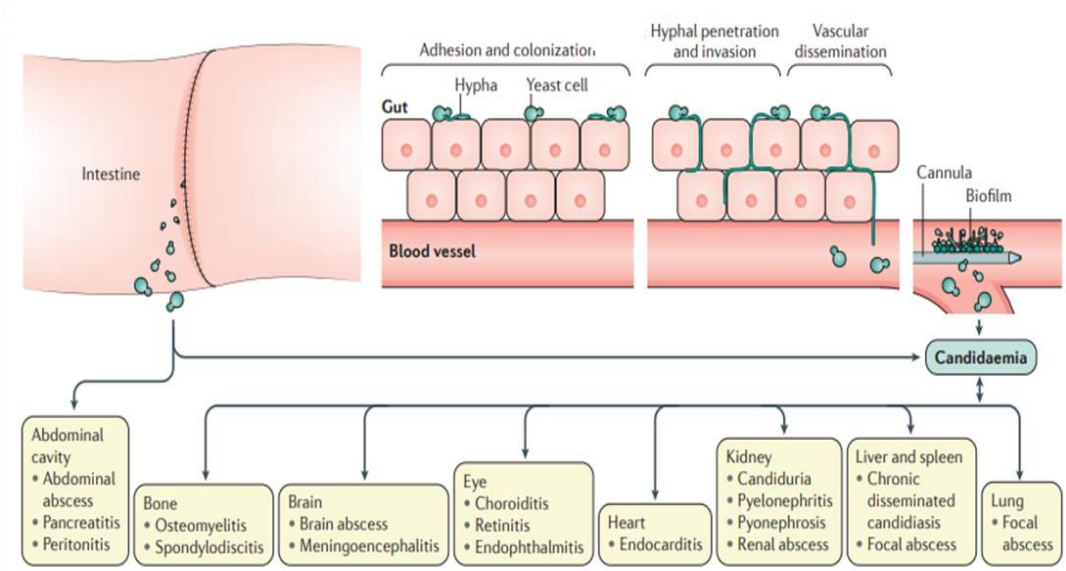


Figure I.8. Model proposed by Pappas *et al.*, 2018 of *C. albicans* colonization of the gut. Under normal conditions, *C. albicans* colonize the human gut and adheres to the intestinal epithelial cells asymptomatically. Nonetheless, through hyphal formation or tissue damage, it can cross the epithelial layer and develop infections throughout most organs of the human host. Figure taken from Pappas *et al.*, 2018[268].

Vulvovaginal candidiasis (VVC) is defined as a superficial symptomatic vaginitis (inflammation of the vagina) caused by infection with *Candida* spp. Common symptoms are vulval itching and abnormal vaginal discharge (which may be minimal, a "cheese-like" material, or a watery secretion)[273]. VVC can be classified into uncomplicated and complicated infections. Uncomplicated cases are characterized by fewer than four episodes per year with mild to

moderate severity, while complicated cases are characterized by recurrent infections (more than four per year) resulting in a condition known as recurrent vulvovaginal candidiasis (or RVVC)[274-276]. Fungal infections of the vulva and vagina are estimated to be the second most common cause of vaginal dysbiosis, after bacterial vaginosis[273, 277]. Around 75% of women experience at least one episode of vulvovaginal candidiasis during their life-time and around 50% of women will develop at least two episodes during their reproductive age[278]. *C. albicans* was estimated to be responsible for 85% to 90% of all VVC cases, with the remaining being attributed to *C. glabrata*, *C. krusei*, and *C. tropicalis*[250, 279, 280]. Around 5 to 8% of VVC cases are estimated to be recurrent, and *C. glabrata* and other NACS are isolated from 10 to 20% of these cases[278, 281]. Host-related risk factors that increase the probability of VVC occurrence include pregnancy, hormone replacement therapy, uncontrolled diabetes mellitus, immunosuppression, and the use of antibiotics or glucocorticoids[250, 256], while behavioural risk factors for VVC include the use of certain contraceptives (such as oral contraceptives and intrauterine devices), spermicides and condoms, as well as certain sexual, hygienic, and clothing habits[250, 256, 282].

The observation that the same *Candida* species identified as colonizers are also those capable of overgrowing suggest that these cells are well equipped with mechanisms that allow them to thrive in this environment, increasing their competitiveness. Indeed, this has been investigated, in particular the ability of *Candida* cells to evade and avoid the host immune system. A common strategy of fungal pathogens to avoid interaction with immune cells is the masking of immunostimulatory components located at the cell wall as a mean to avoid recognition and macrophage activation and/or phagocytosis[283, 284]. Yeasts typically have cell walls made up of chitin, β-1,3-glucan, and β-1,6-glucan, attached to an external layer composed of extensively mannosylated proteins and phospholipomannan[285, 286]. β-1,3-glucan is an essential component in immune recognition being recognized by the dectin-1 receptor present in macrophages[287]. The capacity of masking the β-glucan was first demonstrated for *C. albicans*, with the outer mannan layer shielding the β-1,3-glucan and resulting in low dectin-1 reactivity[288]. Only when the cell wall is disrupted, and β-1,3-glucan becomes exposed, does dectin-1 binding enable macrophage activation and the production of cytokines, including TNF-α[288]. Notably, lactate in the environment was found to trigger β-1,3-glucan masking[289]. In specific, it was found that exposure of *C. albicans* to lactate triggers a signalling pathway that involves the recruitment of the evolutionarily conserved receptor Gpr1 and the transcription factor Crz1[289]. In *C. glabrata* mutants lacking cell surface-proteases (yapsins) were also found to induce a stronger inflammatory response prompted by macrophages, attributable to a higher accessibility of the immune cells to the cell wall components[290-292]. Besides these evasion mechanisms, *Candida* cells also appear to have evolved adaptative responses upon internalization in the macrophage after phagocytosis. In specific, it has been found that *C. glabrata* subverts phagolysosome maturation inhibiting various markers of the phagolysosomal stage, including cathepsin D, proteolytic activity, and lysosomal fusion[293, 294]. Moreover, despite a potent acidification of phagosome prompted by a proton pump V-ATPase, internalized yeast cells retained their viability sustaining the acidification[293, 294]. *C. albicans* cells have also been shown to disrupt the

lysosomal system of macrophages in mice by delaying phagosome maturation and acidification[295, 296]. Macrophage antimicrobial response is also associated with the production of reactive oxygen species (ROS), which is driven by the macrophage NADPH oxidase complex generating superoxide, hydroxyl anions and hydroxyl radicals.[297]. Studies on human and murine macrophages have shown that *C. glabrata* suppresses ROS production undertaken by these phagocytes[293, 298]. Additionally, *in vitro*, *C. glabrata* superoxide dismutase Sod1 was shown to impair the antimicrobial action of macrophages[299] and a catalase *CTA1*-reporter gene was found to be induced after macrophage phagocytosis[300].

The very high adhesiveness of *Candida* cells to biotic and abiotic surfaces is one of the more important virulence factors of these species that strongly contributes to the establishment and persistence of infections[301]. Indeed, *Candida* species are capable of efficiently adhere to vaginal epithelial cells[302] but also to intrauterine devices[303]. The adhesion process to these biotic and abiotic surfaces is mediated by favourable cell surface physicochemical properties and by a set of cell surface adhesins[304]. Adhesins recognize various ligands frequent in the host such as serum proteins as well as components of the extracellular matrix (e.g. laminin, fibronectin, collagen, vitronectin and entactin), also facilitating binding to abiotic surfaces through hydrophobic interactions[305]. Adhesins are better characterized in *C. albicans* that encompasses a major group encoded by the agglutinin-like sequence (ALS) gene family that includes eight members (ALS1-7, 9)[306]. In *C. glabrata*, most adhesins belong to the EPA gene family, which encodes at least 23 different adhesins, including *EPA1*, *EPA6* and *EPA7*[307]. Consistently, these three adhesins were found to be expressed in *C. glabrata* during reconstituted human vaginal epithelium (RHVE) infection, hinting for their critical role in the infectivity of this species, specially of *EPA1* which was more expressed than the others[308]. The study of adhesins in other NACS is limited, although, it was demonstrated that they are required for *C. parapsilosis* and *C. tropicalis* adhesion to biotic and abiotic surfaces[309-311].

One of the most important virulence traits of *C. albicans* is hyphae formation. *C. albicans* and *C. dubliniensis* can form hyphae and/or pseudohyphae, *C. parapsilosis* can generate pseudohyphae, *C. tropicalis* pseudohyphae and possibly true hyphae and *C. glabrata* grows only as budding yeasts[301]. Filamentous forms provide mechanical strength, enhanced colonization and invasion of host tissues and increased protection against phagocytosis[301]. Importantly, hyphae formation was shown to be crucial for tissue invasion, demonstrated by the inability of *C. albicans* strains unable to form hyphae to invade tissues[312]. Hyphae formation in *C. albicans* requires phospholipase D (encoded by *PLD1*)[313] as well some secreted aspartyl proteinases (SAP) genes (*SAP4-6*)[314]. Notably, *SAP4* and *SAP5* were associated with hyphae formation during vaginal candidiasis in mice[315]. Furthermore, it was demonstrated during RHVE infection *C. albicans* had increased expression of the hyphal wall protein (*HWP1*) gene, which is a specific hyphae-adhesin responsible for a covalent attachment of the yeast to host epithelial cells, providing evidence of the role of hyphal formation on tissue adhesion and invasion[308].

The capability to form biofilms is another important factor for the dissemination and virulence of *Candida* spp. Biofilms are highly organized structured communities of microorganisms, irreversibly attached to a surface and enclosed in a self-produced extracellular matrix[316]. The ability to form biofilms provides *Candida* spp. advantages over their planktonic cells, including increased resistance to antifungals, to host defence mechanisms and to physical and chemical stresses occurring in the environment[317]. Additionally, biofilm cells exhibit metabolic cooperation, community-based regulation of gene expression and the ability to withstand the competitive pressure from other organisms[318]. Biofilm formation process is better characterized in *C. albicans*, being divided into three stages of development that include the adhesion of the yeast cells to the surface (early stage); the differentiation of the yeast cells into hyphae cells (intermediate stage); and increased matrix production, which is the maturation phase[319]. Nonetheless, the ability to form biofilms in *Candida* is highly dependent of the species, strain and even of the environmental conditions thus remaining very complex to establish the extent at which it contributes for increased virulence of *Candida* in the vaginal tract[320, 321]. Importantly, *C. albicans* was demonstrated to form biofilms on vaginal epithelium[322, 323], being dependent of *BCR1* and *EFG1* genes, and correlated with the use of intrauterine devices (IUDs) which contributed to the increased risk of VVC[324].

To adhere and invade host epithelial cells, *Candida* spp also requires several secreted hydrolytic enzymes crucial for adhesion, tissue penetration, invasion and destruction of host tissues[325]. These enzymes include secreted aspartic proteases (SAPs), phospholipases, lipases and haemolysins and are essential for *Candida* virulence[301]. SAPs play a role in enabling attachment to host tissues and causing damage, and are associated with alterations in the host immune response[326]. In *C. albicans* were identified 10 SAP genes (SAP1-10)[327], while in *C. parapsilosis* and *C. tropicalis* were identified three and four of such genes, respectively[328, 329]. In *C. glabrata* no obvious SAP genes could be identified by analysis of its genome[330], albeit in one study it was shown the ability of a strain to produce proteinases, although it failed to elucidate the type[331]. An interesting aspect of SAPs is that contrary to other proteinases their activity is maximal under acidic conditions ($pH \leq 4.0$)[325, 332]. This characteristic is of most importance when discussing virulence of *Candida* in the vagina due to its intrinsic acidity[333]. Consistently, several studies have reported higher expression of SAPs and higher proteinase activity prompted by *Candida* strains isolated from women with VVC, compared to strains recovered from asymptomatic women, suggesting a role for these enzymes in the development of VVC[334]. Phospholipases exert their function by hydrolysing the ester bonds in glycerophospholipids contributing to membrane damage and improving adhesion of *Candida* to host tissues[335]. As with SAPs, the expression of phospholipases is strongly dependent of the species and even the strain, with *C. albicans* having higher expression compared with NACS[336]. On top of that, it seems that phospholipase expression is dependent of the environment, being more expressed in strains obtained from the vagina compared with those recovered from the respiratory tract or from the skin[337]. In *C. albicans* seven phospholipase encoding genes were identified (*PLA*, *PLB1-2*, *PLC1-3* and *PLD1*)[338], with *PLB1*

and *PLB2* being significantly more expressed in strains recovered from women with VVC, compared with strains recovered from asymptomatic carriers[339]. Notably, *C. albicans* cells recovered from infected RHVE showed increased expression of *PLB* and *PLD* gene families[308].

I.4.1. Available therapeutic approaches for the treatment of candidiasis

The development of therapeutic methodologies for treating candidiasis is difficulted by the similarities existing between the yeast and the host cells, both eukaryotic. Currently, the treatment of *Candida* infections involves the administration of antifungals belonging to four major drug classes: azoles (e.g., fluconazole), polyenes (e.g., amphotericin B), echinocandins (e.g., caspofungin) and fluoropyrimidines (5-flucytosine (5-FC)) (Figure I.9).

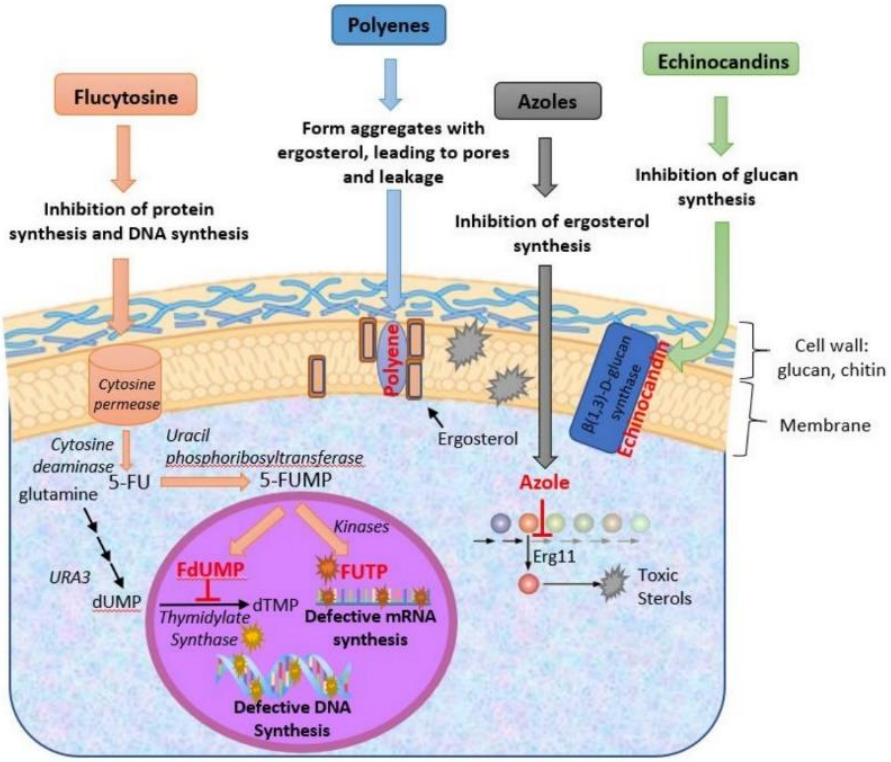


Figure I.9. Schematic representation of the known mechanisms of action of the different classes of antifungals available for the treatment of candidiasis. Abbreviations: 5-FU - 5-fluorouracil; 5-FUMP - 5-fluorouridine monophosphate; FdUM - 5-fluorodeoxyuridine monophosphate; FUTP - 5-fluorouridine triphosphate. Figure taken from Salazar *et al.* 2020[259].

The largest family of antifungals used to treat *Candida* infections is the azoles. Clotrimazole and miconazole were the first azoles to be approved for clinical use in 1969, followed by ketoconazole in 1981[340]. These three drugs are classified as imidazoles due to the presence of an imidazole ring in their structure. However, the use of clotrimazole and miconazole as antifungals was restricted due to their inhibitory effect on human hepatic CYP enzymes[341]. To address this issue, in the early 90s, fluconazole and itraconazole were introduced, both belonging to the triazole family and, compared to imidazoles, exhibiting improved pharmacokinetic profiles,

broader antifungal activity, and reduced inhibitory effects against the human CYP450 system[340]. Voriconazole was introduced in the early 2000s and demonstrated higher activity against the more azole-resistant NACS than fluconazole or itraconazole[340]. Currently, imidazoles are mainly used to treat superficial candidiasis, while triazoles are preferred to treat invasive candidiasis[340]. Regardless of their family, azoles function by inhibiting the activity of the lanosterol-14α-demethylase enzyme, encoded by the *ERG11* gene, which is involved in ergosterol biosynthesis (Figure I.9)[342]. As a result, azole-treated fungal cells accumulate toxic sterols in the plasma membrane, significantly impacting its permeability[342].

Polyenes, including amphotericin B, which was the first antifungal agent developed to treat disseminated candidiasis[343] and nystatin, mostly used to treat oral infections[344], are also widely used against *Candida* infections. These are macrocyclic organic molecules composed, in most cases, by a 20-40 carbon macrolactone ring conjugated with a d-mycosimine group[345]. Due to the amphipathic nature of these molecules, they can bind to the lipid bilayer of the membrane and form a complex with the ergosterol, producing pores that result in cell permeability, leakage of cytoplasmic contents, oxidative damage and, ultimately, cell death (Figure I.9)[346]. Despite its potent antifungal effects against *Candida*, the use of amphotericin B is limited due to its nephrotoxicity resulting from its ability to bind to the cholesterol present in human cells[347, 348]. Although safer formulations have been developed, mostly based on the use of liposomes, the high cost of amphotericin B remains a significant obstacle, being mostly used as a second-line therapy[348].

In recent years, the only newly discovered class of antifungals was the echinocandins[349], commercially available in three forms: caspofungin, anidulafungin, and micafungin. More recently, rezafungin and biafungin have also been described, but their efficacy in the clinical setting is still being assessed in clinical trials. Nonetheless, compared to the already available echinocandins, rezafungin and biafungin have shown higher activity, lower toxicity, and fewer drug interactions[350-352]. Echinocandins inhibit the catalytic subunits of (1,3)-β-D-glucan synthase, which is essential for cell wall synthesis, resulting in no elongation of (1,3)-β-D-glucans in fungal cells exposed to these antifungals (Figure I.9)[342]. This renders the fungal cells highly susceptible to lysis[342]. Although *C. parapsilosis* is intrinsically less susceptible, echinocandins have shown efficacy against all *Candida* species[348]. Due to their safety profile and fungicidal activity, echinocandins are frequently used as the primary treatment for invasive candidiasis[348].

Finally, the most commonly used fluoropyrimidine for the treatment of candidiasis is 5-flucytosine (5-FC), which enters the fungal cells through cytosine transporters and is subsequently metabolized via the pyrimidine salvage pathway to 5-fluorouracil (5-FU), the active form of 5-FC[342, 353]. Once 5-FU is incorporated into RNA, it causes premature chain termination and inhibits the activity of thymidylate synthase, an enzyme essential for DNA synthesis (Figure I.9)[342, 353]. *Candida* species are susceptible to 5-FC, with the exception of *C. krusei*. Although mammalian cells lack the enzymes required to convert 5-FC into 5-FU[342], gut bacteria are capable of efficiently undertaking this conversion[353], which explains the

reported toxic effects associated to 5-FC therapy. Due to its potential for toxicity, 5-FC is administered in low concentrations and in combination with other antifungal agents[348].

Depending on the severity of the infection several therapies based on the above described antifungals are utilized, alone or in combination, using topical or oral formulations (as detailed in Table I.3). A mild infection is mostly treated using short-term local therapy or using a single-dose oral treatment. In the case of VVC, this approach is effective in suppressing the majority of uncomplicated cases[274, 275]. Typically, it takes up to 3 days to treat VVC using local azoles, resulting in efficacy rates of 80 to 90% in most cases[354]. Commonly used azoles in these circumstances include fluconazole, clotrimazole, and miconazole[273]. Another approach besides the local application of azoles in the infected area is the oral treatment with a single dose of 150 mg fluconazole. Efficacy of this treatment was evaluated in a clinical trial where the authors compared it to one week application of clotrimazole vaginal suppositories[355]. The results obtained showed similar effectiveness for these two treatments that reached approximately 80% of cure in patients after seven days of antifungal administration[355]. In contrast, treatment of RVVC, requires a more prolonged therapy. In these cases, it is recommended that patients take three times a dose of oral fluconazole with a gap of 72h, or undergo treatment with local azoles daily for at least 1 week[274]. In Sobel *et al.*, 2001 the authors compared a single dose and a two-dose ingestion of oral fluconazole in women with complicated VVC, concluding that the two-dose treatment was better at treating disease[356]. Moreover, another study by Sobel *et al.*, 2004 evaluated the effectiveness of fluconazole in treating recurrent VVC. Here a total of 387 patients suffering from recurrent VVC were randomly divided into study and placebo groups. After initial treatment with 150 mg doses of fluconazole every 72h, the study group received weekly doses of 150 mg of fluconazole for 6 months while the placebo group did not receive any dose of fluconazole. After 6 months, 90.8% of the women that received the fluconazole doses remained disease-free, while among the women receiving the placebo only 35.9% were found not to develop the disease[357]. The average recurrence of VVC was approximately 4 months in the placebo groups whereas in the fluconazole group was around 10 months[357]. However, the treatment of VVC with azoles is less effective against NACS and it is also important to note that prolonged treatments in prophylactic regimes are likely to increase selective pressure for resistance. Other commercially available alternatives to treat *Candida* infections are the use of polyenes. The effectiveness of nystatin applied locally to the vagina, compared with oral fluconazole, in a cohort of 293 RVVC patients demonstrated that both methods were effective in treating the disease[358]. Additionally, nystatin was shown to have the advantage of being efficient in treating infections caused by *Candida* strains resistant to azoles[358]. Another study assessed the effectiveness of local administration of 50 mg amphotericin B in a suppository form for two weeks in women with VVC caused by NACS, achieving a success rate in treating the infections that was circa 70%[359]. Limited studies have been done to investigate the effectiveness of echinocandins in treating VVC, contrasting with their application in the context of oropharyngeal, oesophageal, and invasive candidiasis were they had been found to be quite successful[360]. The use of topical treatments involving flucytosine and boric acid to treat VVC

had also been determined, revealing that administration of 600 mg boric acid intravaginally for 14 to 21 days was effective in 64 to 71% of the cases, while a 14 days therapy using flucytosine was effective in treating 90% of the cases that were not susceptible to boric acid[361].

Table I.3. Common therapies used to treat vulvovaginal candidiasis (VVC). Table described the drug used, formulation (topical or oral agents) and the dosage for each treatment. Table taken from Bondaryk *et al.*, 2013[362].

Drug	Formulation	Dosage regimen
Topical agents		
Butaconazole	2% cream	5 g for 3 days
Clotrimazole	1% cream	5 g for 7 to 14 days
	100 mg vaginal tablets	1 tablet for 7 days
	100 mg vaginal tablets	2 tablets for 3 days
	500 mg vaginal tablets	Single dose of 1 tablet
Miconazole	2% cream	5 g for 7 days
	100 mg vaginal suppository	1 suppository for 7 days
	200 mg vaginal suppository	1 suppository for 3 days
	1200 mg vaginal suppository	Single dose of 1 suppository
Econazole	150 mg vaginal tablets	1 tablet for 3 days
Fenticonazole	2% cream	5 g for 7 days
Tioconazole	2% cream	5 g for 3 days
	6.5% cream	Single dose of 5g
Terconazole	0.4% cream	5 g for 7 days
	0.8% cream	5 g for 7 days
	80 mg vaginal suppository	80mg for 3 days
Nystatin	100 000 U vaginal tablets	1 tablet for 14 days
Oral agents		
Ketoconazole	400mg twice a day	For 5 days
Itraconazole	200mg twice a day	For 1 day
	200mg	For 3 days
Fluconazole	150mg	Single dose

The use of lactobacilli-based probiotics is another potential approach to treat *Candida* infections. The pursue of this line of treatment has been gaining traction as it increases the need of finding novel therapeutic targets besides those targeted by the above described antifungals to overcome the observed emergence of resistant strains[363, 364]. Presently, the consensual definition of probiotics is “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. This definition was recommended by the International Scientific Association for Probiotics and Prebiotics which retained the probiotic definition given by the Food and Agriculture Organization of the United Nations and World Health Organization (WHO). The most used probiotics belong to *Lactobacillus* and *Bifidobacterium* genera, but other species (such as those belonging to the genus *Bacillus*, *Streptococcus*, or *Saccharomyces*) can also be used[365-368]. Several lactobacilli species have already been used, alone or in combination with other drugs, to treat candidiasis in patients suffering from acute or severe VVC (Table I.4). The results obtained show that women with acute VVC receiving only antifungal

therapy, compared with women receiving antifungal therapy and a vaginal probiotic containing *L. acidophilus*, *L. rhamnosus*, *S. thermophilus*, and *L. delbrueckii* subsp. Bulgaricus, have more clinical complaints[369]. More recently, Xin Yee Ang *et al.*, 2022 tested the effectiveness of a probiotic containing *Lactobacillus helveticus* LA25, *L. delbrueckii* subsp. lactis LDL114, *L. plantarum* LP115, *L. fermentum* LF26, *L. paracasei* LPC12 and *L. rhamnosus* LRH10 (SynForU-HerCare; two capsules/day of 9.5 log CFU/capsule) and concluded that it reduced the abundance of *Candida* cells in the lower and higher vaginal region, while increasing the abundance of *L. crispatus* in the lower vaginal region and of *L. jensenii* in the cervicovaginal region[370]. These changes in the microbiome were also accompanied by a shortening in the period of inflammation[370]. In the same direction, the intake of vaginal tablets containing *L. plantarum* I1001 was associated to a three-fold reduction in the risk of VVC recurrence for 91.3% of women after six months when administrated as a follow-up therapy after clotrimazole treatment[371]. Additionally, a study using a *L. acidophilus* based probiotic without prior fluconazole treatment proved successful in treating VVC[372]. Briefly, in this study patients were divided into two groups: the first received a single dose of 150 mg fluconazole before probiotic administration, while the second one received a placebo before the administration of the probiotic[372]. Both groups showed similar effectiveness in treating VVC after 30 to 35 days after starting the treatment[372]. Another study also investigated the effectiveness of a probiotic containing *L. rhamnosus* DSM 14870 *and L. gasseri* DSM 14869 in treating VVC, reporting that women who received both fluconazole and probiotics achieved cure rates of 100% and 89% after 6 and 12 months, respectively. In contrast, those who received only fluconazole had cure rates of 100% and 70% after 6 and 12 months, respectively; thus indicating that the probiotic cocktail used improved (albeit slightly) the ability of fluconazole to prevent recurrence of VVC[373]. There are several other clinical trials that examined the potential of probiotics to treat infections caused by *Candida*, with the majority of them concluding that probiotic supplementation attenuates infection symptoms, reduces *Candida* abundance and/or reduces the recurrence of VVC when used alone or in combination with antifungal drugs[374-377], reinforcing the notion that probiotics, mostly formulated using lactobacilli, can be quite useful tools to treat VVC (Table I.4).

Table I.4. Studies reporting clinical trials on the use of lactobacilli-based probiotics against VVC (Part 1). The table described the probiotic strain, the subjects of the clinical trial, the therapy duration, the groups compared, the study and the main outcomes of the trial. Abbreviations: VVC – vulvovaginal candidiasis; RVVC – recurrent vulvovaginal candidiasis.

Probiotic cocktail	Subjects	Duration of therapy	Groups	Main outcomes	Study
L. acidophilus*, *L. rhamnosus*, *L. delbrueckki* subsp. bulgaricus and *S. thermophilus	416 patients suffering from acute VVC	10 days	Antifungal treatment (fluconazole and fentinazole) Antifungal treatment (fluconazole and fentinazole) + probiotic administration	The probiotics increased the effectiveness of the antifungal therapy. Briefly, in patients receiving probiotics, clinical complaints were reduced by 15%, the number of *Candida* hyphae and spores reduced by 31% and 26% respectively, and the abundance of *C. albicans* reduced by 32%, while lactobacilli abundance increased 7%	[369]
***L. helveticus* LA25, *L. delbrueckii* subsp. lactis LDL114, *L. plantarum* LP115, *L. fermentum* LF26, *L. paracasei* LPC12 and *L. rhamnosus* LRH10**	78 pregnant women suffering from VVC	8 weeks	No probiotic treatment after VVC treatment with 200 mg of clotrimazole Probiotic administration (2 capsules per day) after VVC treatment with 200 mg of clotrimazole	The probiotics decreased the abundance of *Candida*, promoted an increase in indigenous lactobacilli species and decreased vaginal inflammation	[370]
L. plantarum	55 patients suffering from acute VVC	2 months	Clotrimazole treatment Clotrimazole treatment and vaginal probiotic administration 3 times per week	The use of probiotics after clotrimazole treatment reduced the risk of symptomatic recurrence three-fold over 3 months. Recurrence after 6 months was 27.2% in the probiotic group, compared with 65.1% in the azole treatment only group	[371]
L. acidophilus	80 married women suffering from acute VVC	65 days	Probiotic treatment after 1 dose of 150 mg fluconazole Probiotic treatment without fluconazole administration	Probiotic administration treatment had an effect similar to that of probiotic and fluconazole in most VVC-associated symptoms, however, it was less effective in preventing recurrence	[372]

Table I.4. Studies reporting clinical trials on the use of lactobacilli-based probiotics against VVC (Part 2). The table described the probiotic strain, the subjects of the clinical trial, the therapy duration, the groups compared, the study and the main outcomes of the trial. Abbreviations: VVC – vulvovaginal candidiasis; RVVC – recurrent vulvovaginal candidiasis.

Probiotic strain	Subjects	Duration of therapy	Groups	Main outcomes	Study
***L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869**	28 women, with RVVC, suffering from acute VVC	6 months	Antifungal treatment (50 mg every day) Antifungal treatment and probiotic administration	Probiotic supplementation slightly improved the ability of fluconazole to prevent VVC recurrence (19%)	[373]
L. acidophilus*, *B. bifidum* and *B. longum	59 women, with RVVC, suffering from acute VVC	10 days	Treatment with 1 dose of 150 mg of fluconazole Probiotic treatment after 1 dose of 150 mg fluconazole	Probiotic administration after antifungal treatment was effective in treating VVC and reduced the recurrence rate from 35.5% to 7.2%	[374]
***L. fermentum* LF10, *L. fermentum* LF11, and *L. acidophilus* LA02 with arabinogalactan and fructooligosaccharides**	30 women, with RVVC, suffering from acute VVC	2 months	No control/placebo group Treatment with probiotic	Probiotic treatment was effective in treating VVC in 86.6% of the patients. Additionally, only 11.54% of the patients suffered a recurrence	[375]
***L. fermentum* LF10, *L. acidophilus* LA02 with arabinogalactan and fructooligosaccharides**	58 women, with RVVC, suffering from acute VVC	11 weeks	No control/placebo group Probiotic administration after fluconazole treatment	Probiotics decreased recurrence of VVC. After 10-week probiotic treatment, only 14% developed VVC and during the 7-month follow-up only 14.3% of patients suffered a recurrence	[376]
***L. plantarum* P17630**	93 women, with RVVC, suffering from acute VVC	90 days	Placebo treatment Probiotic treatment	Probiotic intake significantly attenuated the VVC associated symptoms (of about 33%) after 90 days compared with the placebo group	[377]

The role of a healthy microbiota and the probiotics potential in maintaining a healthy environment in the gut is also well documented, but this has been mostly studied in the context of inflammatory bowel disease (IBD)[194], coeliac disease[195, 196], allergy[197], cardiovascular disease[198], obesity[199] and gut infections (e.g., *Clostridium difficile infection*)[200-204]. The benefits of administering probiotic cocktails containing *Lactobacillus* spp. (composed by *L. rhamnosus*, *L. acidophilus* and/or *L. reuteri*) and/or *Bifidobacterium*, in effectively reducing *Candida* spp. colonization in the gut had been demonstrated (as detailed in Table I.5). It is worth mentioning that in probiotics containing more than one species (e.g., *Lactobacillus* and *Bifidobacterium*) the anti-*Candida* potential observed could result from a synergy between these organisms. Administration of *L. acidophilus*, *L. rhamnosus*, *B. longum*, *B. bifidum*, *S. boulardi* and *S. thermophilus* was found to reduce the number of patients colonized with *Candida* species after 7 and 14 days, while also reducing the incidence of candiduria (from 37.3% to 17.3%) and from candidemia (from 6.35% to 1.61%) compared with the results obtained in the patient group that was not treated with the probiotic cocktail but with the placebo[378]. Newborns food supplementation with *L. acidophilus*, *B. longum*, *B. bifidum* and *B. lactis* also reduced *Candida* load in the gut, with *C. albicans* being only detected in 12.5% of the subjects taking probiotics, compared with 28.6% in the placebo group[379]. Notably, the presence of *C. glabrata* was only found in the placebo group[379]. Finally, a probiotic cocktail composed of *L. rhamnosus* and *B. animalis* was also found to reduce the levels of *C. albicans* antibodies in schizophrenic patients suggesting a reduction of these yeast in the gut[380]. Additionally, some studies have used probiotics containing only *Lactobacillus* spp., including *L. rhamnosus* and *L. reuteri*. These demonstrated that probiotic treatment with *L. rhamnosus* significantly reduced *Candida* spp. colonization of the gut from 48.8% in the control groups to 23.1% in the probiotic group[381], while a probiotic containing *L. reuteri* was as effective as nystatin in reducing *Candida* colonization of the gut and skin[382].

Table I.5. Studies reporting clinical trials on the use of lactobacilli containing probiotics against gastrointestinal candidiasis. Table described the probiotic strain, the subjects of the clinical trial, the therapy duration, the groups compared, the study and the main outcomes of the trial.

Probiotic strain	Subjects	Duration of therapy	Groups	Main outcomes	Study
L. rhamnosus	80 preterm neonates with a birth weight above 1500 g	12 months	Human milk group Human milk supplemented with *L. rhamnosus* group	Probiotic supplementation significantly reduced *Candida* spp. colonization of the gut from 48.8% in the control groups to 23.1% in the probiotic group	[381]
L. acidophilus*, *L. rhamnosus*, *B. longum*, *B. bifidum*, *S. boulardi* and *S. thermophilus	150 children between 3 months to 12 years who had received broad-spectrum antibiotics	14 days	Placebo Group Probiotic group	Probiotic supplementation reduced the number of colonized patients with *Candida* spp. from 29 to 19 after 7 days and from 34 to 21 after 14 days after treatment. Candeduria incidence reduced from 37.3% to 17.3% and candidemia from 6.35% to 1.61% in the probiotic-treated group compared with the placebo	[378]
L. reuteri	300 preterm infants	From the first feed until death or hospital discharge	Antifungal group (nystatin) Milk supplemented with *L. reuteri* (probiotic group)	*L. reuteri* supplementation is as effective as nystatin in reducing *Candida* colonization of the gut (18.7% compared with 16%) and skin (14% compared with 12%). Invasive candidiasis was detected in two patients of the probiotic group and one patient of the antifungal group	[382]
L. rhamnosus* and *B. animalis	56 patients diagnosed with schizophrenia between 18-65 years	14 weeks	Placebo group Probiotic group	Patients taking a placebo had higher levels of *C. albicans* antibodies compared to those taking the probiotics following a 14-week regimen	[380]
L. acidophilus*, *B. longum*, *B. bifidum* and *B. lactis	112 newborns	6 weeks	Placebo group Probiotic group	Probiotic supplementation reduced *Candida* load in the gut. Only 12.5% of the subjects taking probiotics were colonized by *C. albicans* compared with 28.6% of the placebo group. Additionally, 35.7% of the placebo subject had *C. glabrata* while none of the subjects of the probiotic group had *C. glabrata* in the gut	[379]

Although it is considered that probiotics are valuable in the treatment of VVC and gastrointestinal candidiasis, not all clinical studies on the use of probiotics report the same level of effectiveness, which can be attributable to the variability of the different probiotic strains, differences in the delivery mechanisms and or in therapy details, such as the length or the intervals of probiotic administration. Moreover, the results obtained also suggest that the efficacy of probiotics can be dependent of the severity of the infection and also from the patient's characteristics. Another important note is that most studies conducted with probiotics to treat VVC did not resorted to species considered endogenous to this niche, something that can be attributable to these being considerably less studied, compared with other species that have an already long track-record of use in the probiotics worlds[383, 384]. To date, several rules have been considered when determining if a species could be used as a probiotic. The most relevant rules include: microbial lysates, non-living bacteria and non-colonizing spores cannot be considered probiotics; the microorganisms have to be extensively characterized; probiotics should lack antibiotic resistance genes; and probiotics must survive and colonize the human host and interact positively with the healthy microbiota[385]. Recently, it was discussed the development of a decision tree to be used to classify if a potential probiotic species or product could be used as a probiotic therapy in the human host (Figure I.10)[386]. From this analysis it was concluded that probiotic strains should be well characterized, safe for their intended use, supported by at least one positive human clinical trial and be alive in the product at an efficacious dose throughout shelf life[386]. Taking these guidelines into consideration, some endogenous species of the human host fail to be considered as a potential probiotic due to their poor characterization (e.g., *L. gasseri* and *L. jensenii*). This clearly presents an opportunity for the characterization of these less studied species that could be explored with success to help foster treatments against infections caused by *Candida* species.

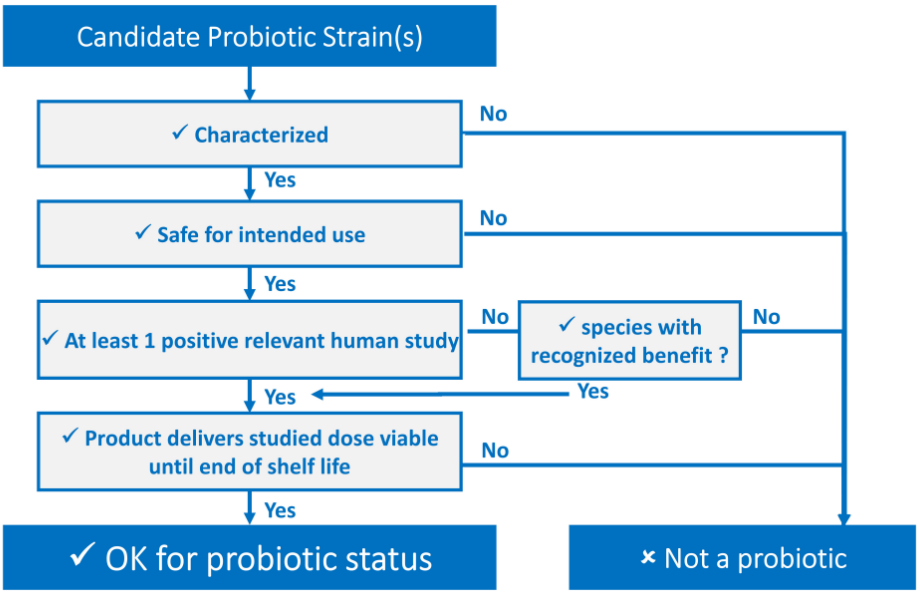


Figure I.10. Proposed decision tree to determine if a candidate probiotic could be considered and used as a probiotic in the human host. Most endogenous species fail because of their poor characterization. Figure taken from Sylvie Binda *et al.* 2020[386].

I.4.2. An overview of the molecular mechanisms underneath the interference effects between lactobacilli and *Candida* cells

As overview in the previous sections a vaginal microflora dominated by lactobacilli has been generally associated with health and reduced activity of vaginal pathogens. Probiotics formulations containing lactobacilli species have been shown to reduce colonization and improve treatment of infections caused by *Candida*, hinting for a possible inhibitory role of lactobacilli against *Candida*. In addition to this, many studies have demonstrated *in vitro* the role of different lactobacilli species (albeit not necessarily those that are part of the vaginal microflora) in inhibiting growth and also relevant traits for pathogenesis of *Candida* in the vaginal tract, such as adhesion, the ability to undertake yeast-hyphae transition; or the capacity to form biofilms. In most cases, however, the so far undertaken studies report only phenotypic observations and do not scrutinize the molecular mechanisms underneath the observed anti-*Candida* effects. Another important note is that in the vast majority of these studies, the anti-*Candida* potential of lactobacilli is evaluated using supernatants obtained from *Lactobacillus* single cultures, that although could be enriched in metabolites with inhibitory activity do not necessarily recapitulate all relevant aspects of this interaction *in vivo* that involves direct cell-cell contacts. Indeed, it was shown that the direct contact of *Lactobacillus* cells with *Candida* results in inhibited growth, adhesion and yeast-to-hyphae differentiation of the yeasts[387].

Studies demonstrated that culture supernatants obtained from *L. gasseri*, *L. crispatus*, *L. rhamnosus*, *L. vaginalis* and *L. fermentum* single cultures, effectively reduced biofilm formation and adhesion to vaginal epithelial cells of *C. albicans* but did not reported whether the same was obtained when these *Lactobacillus* cells were directly co-cultivated with *Candida*[218, 388-391]. Similarly, powerful inhibitory effects had been obtained using supernatants obtained from different vaginal lactobacilli clinical strains[390, 392-394]. Importantly, an exhaustive study of the broth obtained from single cultivation of lactobacilli was unable to correlate the anti-*Candida* properties of these supernatants with the production of lactic acid which is the major fermentation by-product of lactobacilli, meaning that lactic acid is most likely not responsible for the inhibition of *Candida* cells[390]. A more recent thorough analysis, exploring MS-based metabolomics, led to the identification of a small molecule, 1-acetyl-β-carboline (1-ABC), shown to be produced by several lactobacilli (*L. reuteri*, *L. rhamnosus*, *L. helveticus*, *L. casei*, *L. brevis* and *L. plantarum*), that under laboratory conditions was found to blocks the *C. albicans* yeast-to-hyphae transition by inhibiting the Yak1 protein kinase[395]. These findings were very important considering the essential role of Yak1 in prompting biofilm formation by *C. albicans* cell[396], however, it is not clear whether this molecule is also produced in conditions more similar to those observed in the context of human infections, nor the underneath biosynthetic pathway was elucidated preventing the identification of producing and non-producing lactobacilli species[395]. Moreover, was identified in the supernatant of various species of *Lactobacillus* significant amounts of amino acids and derivatives (e.g., glycine, histidine, valine, among others), organic acids (acetate, lactate and

pyruvate), sugars (fructose, galactose, glucose, ribose and myo-inositol), sn-glycerol-3-phospocholine orotate and uracil[397]. Additionally to these metabolites, smaller concentrations of 4-hydroxyphenylacetate, 4-hydroxybenzoate, acetone, hydroxyacetone and ethanol, among many others were also identified[397]. Among these, high concentrations of histidine, acetate and pyruvate and low concentrations of glycine, lactate and fructose were associated with supernatants with high anti-*Candida* potential[397]. Another example includes the study of the spent growth medium of *L. rhamnosus*, which was found to be highly enriched in intermediate metabolites related to pyrimidine metabolism, pantothenate and CoA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, beta alanine metabolism and thiamine metabolism, among others[398]. Interestingly, among the metabolites produced by *L. rhamnosus*, the authors identified phenyllactic acid[399, 400], mevalonolactone[401], 2-hydroxyisocaproic acid (HICA)[402-404], and 3-hydroxyoctanoate[405] which have already been demonstrated to be potential antifungal agents including against *Candida* spp. (such as *C. albicans*, *C. glabrata*, *C. vini*, *C. tropicalis*, *C. krusei*).

Most commonly, the antimicrobial (and necessarily anti-*Candida*) effect of lactobacilli is attributable to the production of lactic acid[333, 406], along with other molecules that might have antimicrobial activity such as hydrogen peroxide[130, 140, 407], bacteriocins[408], biosurfactants[409] and exopolysaccharides[410] (as detailed in Figure I.11). However, the properties of these molecules may not be exactly the same in every niche where they might be produced. For example, the antimicrobial effect of lactic acid, as of all weak organic acids, is largely dependent of the pH which is very different in the vaginal tract (~4) or in the gut (~5 to 7). In the following sections are dissected what can be the eventual contribution of these different factors, when put into context with relevant physicochemical parameters of the vaginal and intestinal gut that might necessarily modulate the anti-*Candida* properties of the residing lactobacilli-populations.

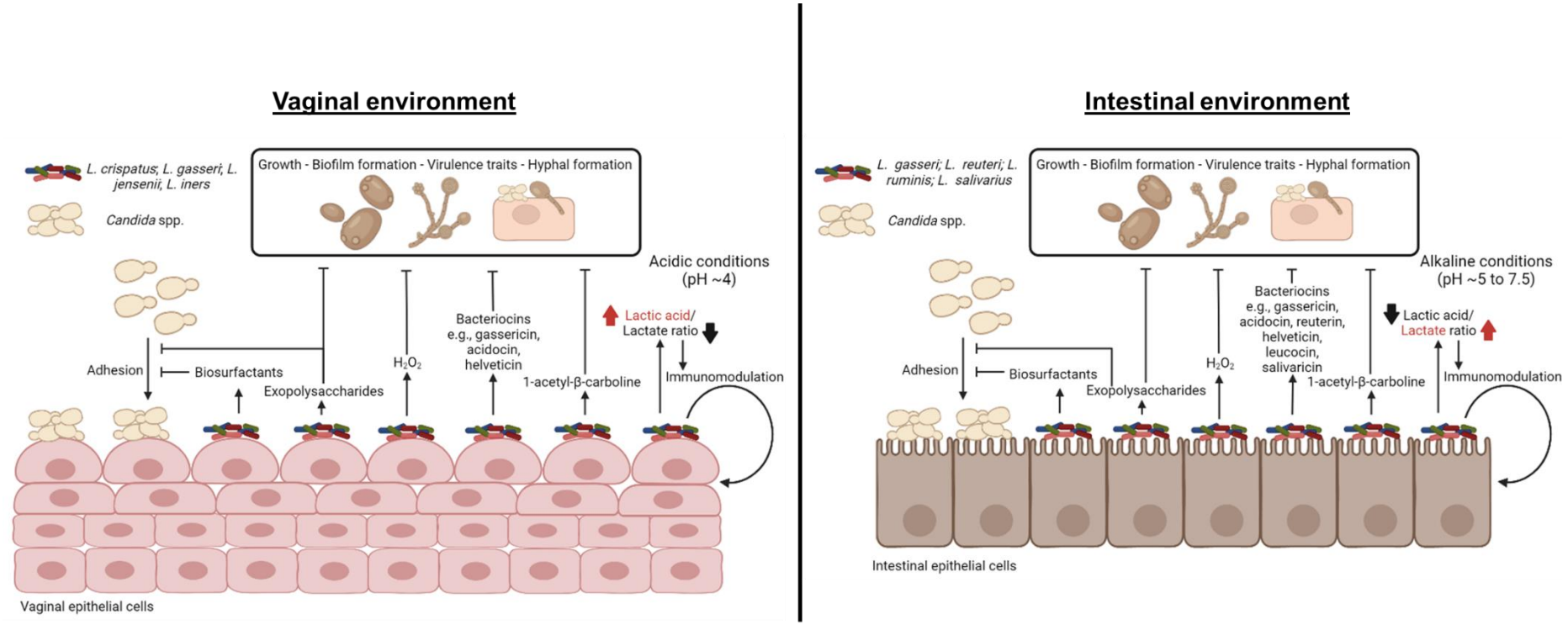


Figure I.11. Proposed mechanisms of how *Lactobacillus* inhibits the adhesion, growth, biofilm formation, hyphal formation and virulence traits of *Candida*. These mechanisms include the secretion of biosurfactants, bacteriocins, H_2O_2, exopolysaccharides and 1-acetyl-β-carboline. Moreover, *Lactobacillus* species have the ability to immunomodulate the host epithelial cells. Although the proposed mechanisms are similar between vaginal and intestinal environments, lactobacilli species and environmental pH are distinct, and thus could differently modulate the anti-*Candida* properties of lactobacilli.

I.4.2.1. Production of lactic acid

The association of a vaginal microflora enriched in lactobacilli with vaginal health has been attributed to the acidification prompted by the bacterium, resulting from their ability to produce lactic acid[96, 97]. Vaginal lactobacilli produce both D- and L-lactic acid in concentrations that can reach 120mM[411-413]. D- and L-lactic acid are not produced by all lactobacilli species, with *L. crispatus* and *L. gasseri* producing both isomers[216, 414], while *L. iners* only producing L- lactic acid[415]. It has been suggested that the domination of *L. iners* in the vaginal microbiome may be less effective in suppressing infections prompted by other pathogens, due to a lower antimicrobial potential of the L-form, in comparison with the D-one[416]. It is worth mentioning that vaginal epithelial cell secretions contain a plethora of compounds (e.g., glycogen, urea, proteins, ions, glycerol, defensins, cytokines and chemokine), including also L-lactic acid[417, 418]. However, studies showed that this L-lactic acid accounts only 15% of the total amount found in the vaginal fluid with the majority being attributed to lactobacilli activity[413, 417]. Although the vaginal lactobacilli are homofermentative, which means they solely produce lactic acid from glucose, some intestinal species (such as *L. reuteri*) perform heterofermentation and thus produce also acetic acid and ethanol as major fermentation products (Figure I.12)[412, 419, 420].

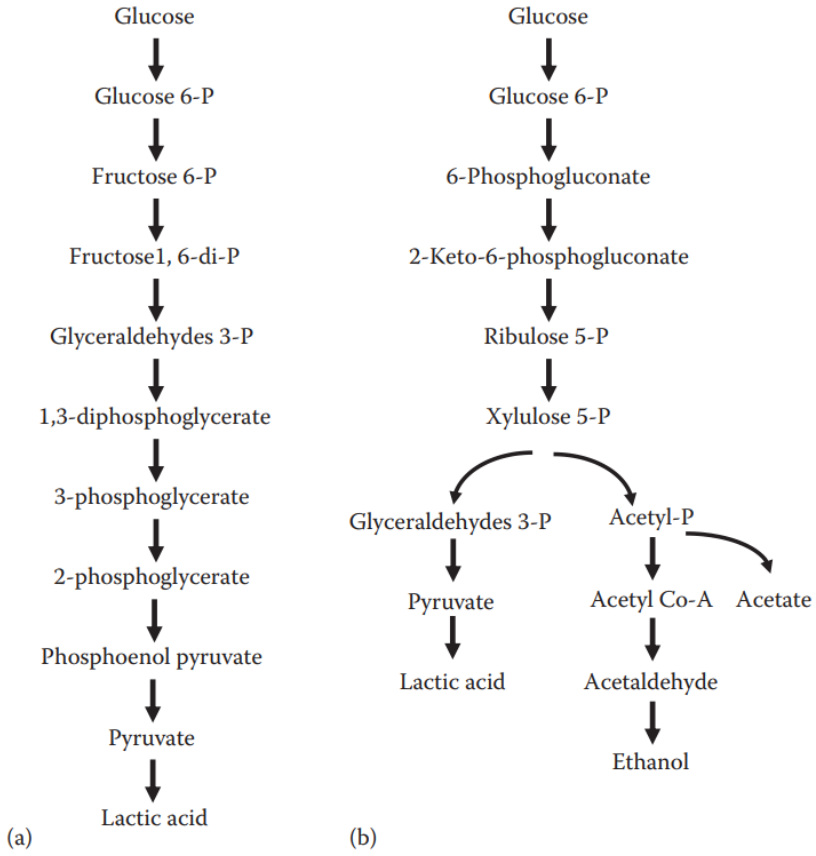


Figure I.12. Brief overview of the metabolic pathways of homofermentative (a) and heterofermentative (b) *Lactobacillus* spp. Homofermentation results mostly in the production of lactic acid as the final by-product while heterofermentation results in the production of lactic acid, acetate and ethanol. Note that the glucose used as the substrate originates from the breakdown of glycogen by α-amylase. Figure taken from Ravinder Kumar *et al.*, 2015[419].

The acidic environment in the vagina is linked with decreased risk of bacterial vaginosis and infections caused by viruses[421]. This correlates well with the poor tolerance of bacteria to acidic conditions, however, *Candida* cells are acidophilic, as most yeasts[422]. Thus, the mere reduction of pH prompted by the vaginal bacteria up to 4 (which is the normal pH found in the vaginal tract) is not likely to pose a significant challenge. It is important to mention that low pH and weak acids exert distinct effects on microbial cells and thus what can be the antimicrobial potential of lactic acid, cannot be confused with the effect of low pH itself. When a strong acid is used, high concentrations of protons (H^+) are produced in the external environment that can affect cell wall structure, alter protein conformation (specially of those protruding from the cell wall) and influence nutrient availability[423]. However, due to poor proton diffusion across the plasma membrane, the inhibitory potential of low pH is well below the one caused by weak acids that dissociate directly in the cytosol[424]. Conversely, at a pH below its pKa the undissociated form (RCOOH) of the weak acid accumulates and can cross the plasma membrane at a rate that will be dependent of its lipophilicity (Figure I.13). Once in the cytosol, the weak acid chemically dissociates, due to the higher cytosolic pH, releasing protons (H^+) and the counter-ion ($RCOO^-$) that cannot cross the hydrophobic membrane bilayer thus accumulating in the cell interior (Figure I.13)[425]. Besides the expected reduction in internal pH, the accumulation caused by the weak acid counter-ion also impacts the lipid organization and function of cellular membranes, affecting the activity of proteins embedded therein and, ultimately, causing nonspecific permeabilization[426-428]; and it can also cause the reduction (and in the limit total dissipation) of the electrochemical potential maintained across the membrane that is essential for secondary transport (Figure I.13)[426-428].

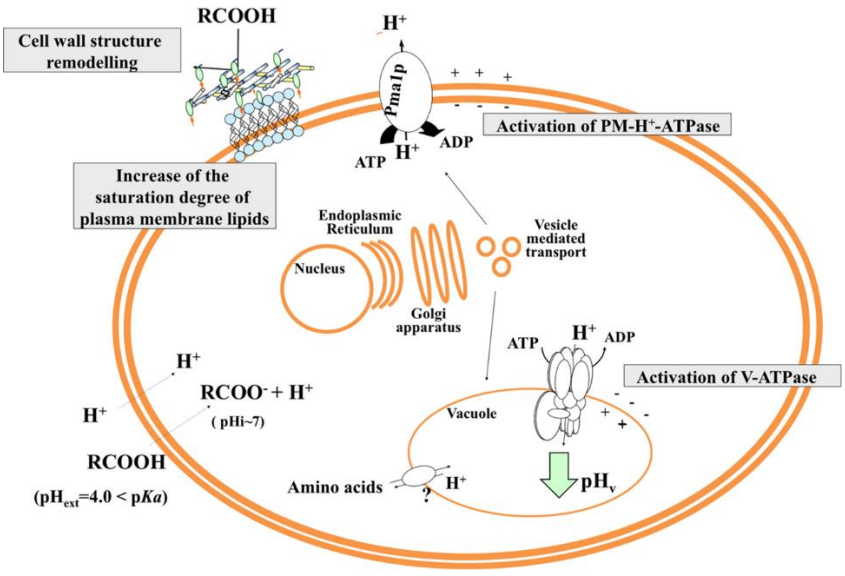


Figure I.13. Mechanistic model describing the toxic effects exerted by weak acids over Yeast cells and corresponding adaptive responses that can be triggered to overcome these effects. Firstly, the activity of H^+-ATPases in the plasma and vacuolar membranes is stimulated, contributing to the recovery of internal pH (pHi) towards more physiological levels and facilitating metabolite compartmentalization in acid-stressed cells. Additionally, the reconfiguration of the cell wall structure and plasma membrane lipid composition may reduce the diffusion rate of undissociated weak acids, resulting in a decrease of weak acid-induced damage to the plasma membrane. Figure taken from Nuno Mira *et al.*, 2010[425].

At the expected vaginal pH of ~4, most lactic acid produced will be in its dissociated form (of lactate) as the pKa of lactic acid is 3.86. Thus, only a small fraction of this will be capable of crossing the plasma membrane and inhibit growth. A study undertaken from our laboratory has examined what could be the potential effect of lactic acid in inhibiting growth of *Candida* cells at different pHs and using different concentrations of glucose and oxygen. The results obtained, showed in Figure I.14, clearly show that under the tested conditions lactic acid did not significantly inhibited growth of *C. glabrata* nor of *C. albicans*[429]. Thus, suggesting that the production of undissociated lactic acid per se does not appear to be a main mechanism for the inhibition of *Candida* prompted by vaginal lactobacilli. In line with this idea, a previous study has not found a correlation between the anti-*Candida* activity of culture supernatants of vaginal lactobacilli strains and the amounts of lactic acid present therein[390]. Nonetheless, the presence of lactic acid was found to boost the inhibitory effect of clotrimazole, fluconazole, miconazole and tioconazole against *C. albicans* and, much less significantly, for *C. glabrata*[430]. In the gut, where the pH is in between 5 to 8[431, 432], the accumulation of undissociated lactic acid is expected to be even lower (in the range of 0-7%).

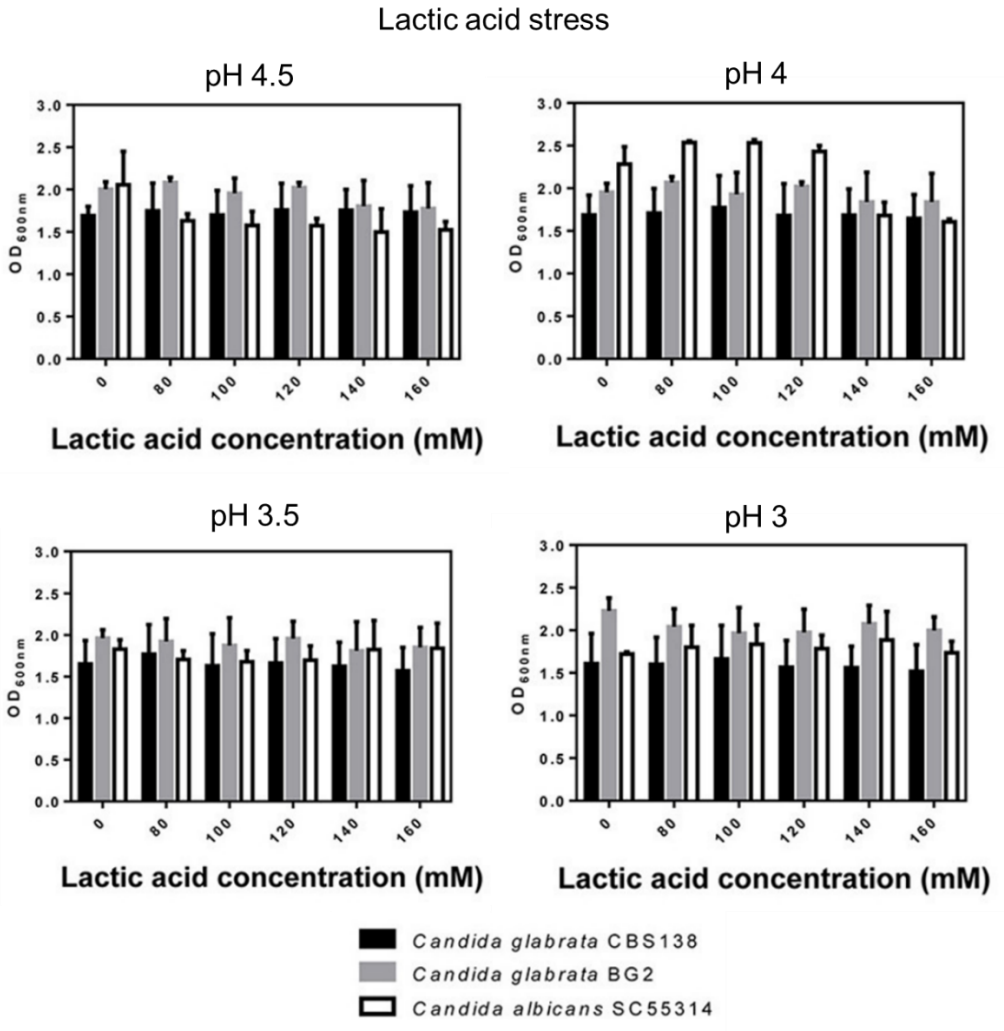


Figure I.14. Lactic acid concentration close to the concentration found in the vaginal tract in a eubiosis state is ineffective in reducing *C. albicans* and *C. glabrata* growth under microaerophilic conditions in minimal medium supplemented with 0.2% glucose in low pH. Adapted from Lourenço *et al.*, 2018[430].

As said above in the vaginal tract, and more prominently in the less acidic environment of the gut, the lactic acid produced by lactobacilli prevails as lactate. *Candida* cells have been shown to be able to consume lactate as a carbon source[433, 434], and this metabolic flexibility is an important virulence trait for these pathogens by allowing them to cope with changes in the panoply of nutrients available in the different niches with different carbon sources. Furthermore, recently it was shown that lactate promotes the evasion of *C. albicans* cells from immune cells by inducing β-glucan masking and reducing macrophage recruitment[289]. Notably, this phenotype was also observed when the yeasts were exposed to culture supernatants of *L. reuteri* and was not found when the yeast cells were exposed to other host-derived metabolites such as acetate, methionine, or proline[289]. The β-glucan masking induced by lactate correlated with reduced uptake of *C. albicans* cells by macrophages *in vitro*, decreased stimulation of TNFα and MIP1α by human macrophages, and diminished recruitment of neutrophils *in vivo*[289]. More detailed molecular analysis revealed that the β-glucan masking is dependent on the Gpr1-Crz1 axis, as schematically represented in Figure I.15. These two findings strongly suggest that *Candida* cells evolved adaptive responses that allowed them to cope with the production of lactic acid, and indirectly of lactate, by lactobacilli in a manner that favours colonization and, eventually, infection.

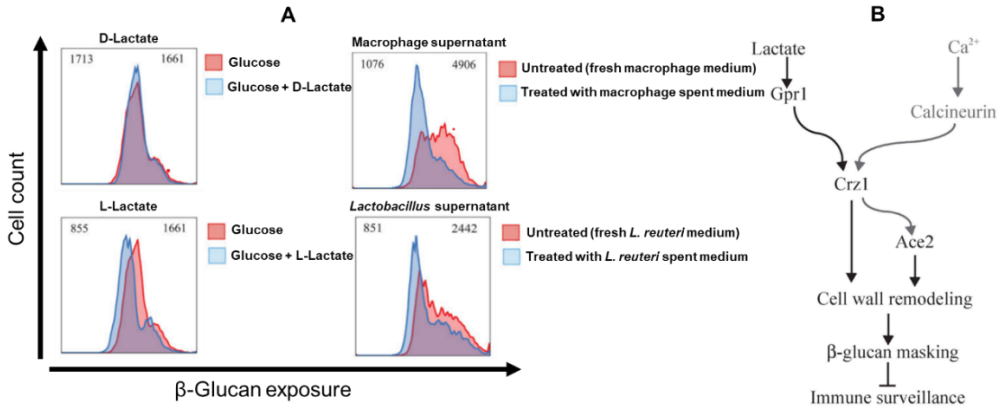


Figure I.15. (A) Flow cytometry analysis of β-glucan exposure. *C. albicans* cells grown in the presence of L-lactate, *L. reuteri* supernatant and macrophage supernatant exhibited reduced β-glucan exposure while D-lactate did not. β-glucan exposure was determined using the median fluorescence intensity (MFI) shown in each graph at the right (glucose/fresh medium grown) and left (glucose+lactate/spent medium grown). **(B)** Proposed model of β-glucan masking in *C. albicans*. L-Lactate is detected by Gpr1, which in turn signals to Crz1 in a calcineurin-independent manner. With the help of Ace2, Crz1 regulates a polygenic response, resulting in the masking of β-glucan on the *C. albicans* cell surface. Adapted from Elizabeth R. Ballou *et al.*, 2016[289].

I.4.2.2. Production of 1-acetyl-β-carboline (1-ABC)

Recently, was demonstrated that several *Lactobacillus* species (*L. rhamnosus*, *L. reuteri*, *L. helveticus*, *L. casei*, *L. brevis* and *L. plantarum*) secrete 1-acetyl-β-carboline (1-ABC) that represses the ability of *C. albicans* to undergo morphogenesis in response to diverse filament-inducing cues[395]. 1-ABC did not, however, reduced viability or growth rate of the fungus when in the yeast form[395]. Using a detailed genetic analysis, the authors successfully identified the target of 1-ABC responsible for the inhibition of *C. albicans* filamentation to be the DYRK (dual-specificity tyrosine phosphorylation-regulated kinase)-family member, Yak1[395]. In brief, 1-ABC

was found to be able to bind to Yak1 protein, resulting in the blocking of signalling transmission to the transcription factor Rob1 protein and, thus, inhibiting the expression of filament-specific genes (Figure I.16)[395]. Since the ability to switch between yeast and filamentous morphologies is a crucial attribute for *C. albicans* pathogenesis[435], the production of 1-ABC can be an important factor in the way by which lactobacilli species can impart virulence of *Candida*. However, the underneath pathway leading to production of β-carboline is not known and therefore it is not possible to determine whether this metabolite could be produced by other lactobacilli and how environmental changes could affect such production. β-carboline compounds are distributed in nature and most derive from plants using pathways that involve a strictosidine synthase that produces a tetrahydro-β-carboline compound through a stereoselective Pictet–Spengler reaction[436, 437]. In bacteria, production of β-carboline compounds has been attributed to specific enzymes in *Marinactinospora thermotolerans* and *Nocardiopsis synnemataformans*, that were found to synthesize 1-acetyl-3-carboxy-β-carboline and 1-acetyl-β-carboline through Pictet–Spengler reactions using l-tryptophan and oxaloacetaldehyde as substrates[438-440]. A completely different mechanism of production of a β-carboline compound was described for the production of kitasetaline by *Kitasatospora setae*[441]. In these cells, the β-carboline core skeleton is formed via successive enzymatic reactions using the KslA, KslB and KslC proteins, and Mca for the synthesis of the N-acetylcysteine moiety of kitasetaline[441]. Interestingly, tryptophan appears to serve as the initial substrate for β-carboline biosynthetic pathways regardless the microbial species[437-441]. However, there was no amino acid sequence homology between these proteins and the proteins of lactobacilli, suggesting that this species may synthesize β-carboline compounds using a still uncharacterized biosynthetic pathway.

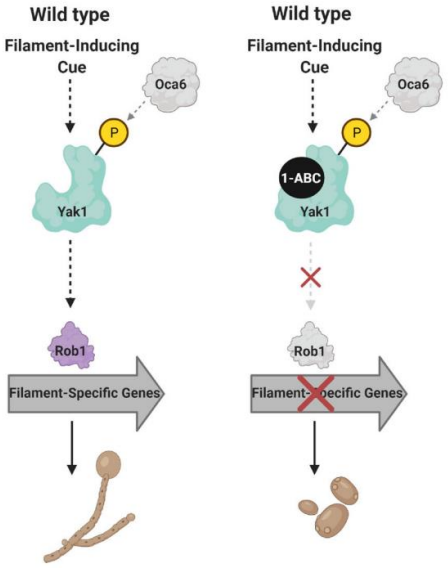


Figure I.16. Proposed model of the mechanisms by which Yak1, Oca6, and Rob1 interact in regulating *C. albicans* hyphal morphogenesis in the absence or presence of 1-ABC. 1-ABC is a strong inhibitor of filamentation specific genes by blocking the action of Rob1 through binding to Yak1. Figure taken from Jessie MacAlpine *et al.*, 2021[395].

I.4.2.3. Bacteriocins

Lactobacillus spp. produce small peptides defined as bacteriocins that have antimicrobial activities and that had been found to provide ecological advantage to producing strains. Numerous studies have identified lactobacilli (e.g., *L. rhamnosus* and *L. acidophilus*) that can produce different bacteriocins (e.g., lactocin 16) with antimicrobial properties against vaginal pathogens (e.g., *G. vaginalis*)[442-444]. These small peptides have a plethora of different structures and physicochemical properties. Although several authors have proposed different classifications for these small proteins, mostly diverging in the number of subclasses, the most well accepted classification divides them into three major different classes[445, 446]: class I, class II and class III. Bacteriocins classified in class I are small (<5 kDa) heat-stable peptides that are highly posttranslational modified and that contain characteristic polycyclic thioether amino acids such as lanthionine, methyl-lanthionine, and unsaturated amino acids such as dehydroalanine and 2-amino isobutyric acid[447]. These can be divided into several different subclasses, including lanthipeptides (type I and type II), cyclic peptides, sanctibiotics, linear azol(in)e-containing peptides and glycocins[446, 448]. Lanthipeptides are characterized by the presence of sulfur-to-β-carbon thioether crosslinks (named lanthionines and (methyl)lanthionines), while cyclic bacteriocins have the feature of having their N- and C-terminals linked by a peptide bond, resulting in a circular molecule. Sanctibiotics are characterized by the presence of a linkage between a cysteine thiol and the α-carbon of another residue resulting in a sulphur-bridged protein, and linear azol(in)e-containing peptides (LAPs) have in their composition various combinations of heterocyclic rings of thiazole and (methyl)oxazole. Finally, glycocins are bacteriocins containing glycosylated residues[446, 448]. Among these types of bacteriocins, the most common examples are nisin and lacticin 3147 (type I and type II lanthipeptides, respectively), enterocin, subtilisin A, streptolysin S and glycocin F, respectively[446, 448].

Class II bacteriocins are heat-stable peptides slightly larger than those classified in class I (<10 kDa) without lanthionine and not-posttranslational modified (beyond the elimination of a leader peptide and the formation of a conserved N-terminal disulphide bridge[446, 448, 449]). Class II is subdivided into IIa, IIb and IIc. Subclass IIa comprises bacteriocins that are monomers and have an N-terminal consensus sequence Tyr-Gly-Asn-Gly-Val-Xaa-Cys, e.g., pediocin PA-1. Subclass IIb includes two-component bacteriocins having two different peptides acting synergistically to generate an antimicrobial effect (e.g. lactococcin G)[446, 448]. Finally, subclass IIc is composed of circular bacteriocins, e.g., gassericin A, that carries two transmembrane segments to enable the formation of pores in the target cells membrane[446-449].

Class III comprise the larger molecular weight bacteriocins (>30kDa) that are not included in any of the classes described above. Bacteriocins from this group have various domains, hence their size, and are heat-sensitive proteins, e.g. helveticin[446-449].

Despite their very distinct structures, the general mechanism by which bacteriocins exert their antimicrobial activity is by forming pores and permeabilizing the cell membrane of the target

cell[450, 451]. While the mechanisms for pore formation are not well understood in general, in the case of nisin A, lacticin and lactococcin this has been more thoroughly investigated. Briefly, nisin (a Class I, type I lanthipeptide) was shown to remove lipid II from its natural location which prompts the insertion of the bacteriocin into the cell membrane resulting in formation of a pore (Figure I.17)[452].

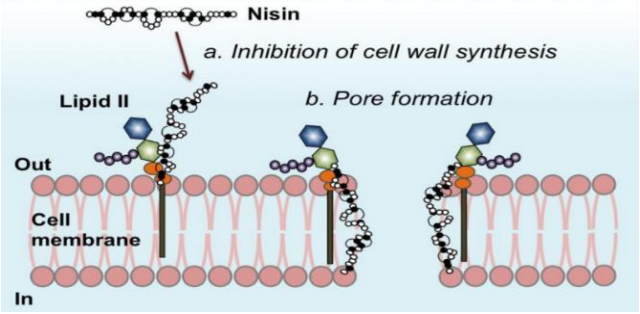


Figure I.17. Proposed model for the lipid II-mediated nisin pore formation. Nisin first binds to the outwardly orientated carbohydrate moiety of lipid II in a 1:1 stoichiometry. The N-terminal segment of the nisin is essential for binding, and a negative surface charge is not necessary. The C-terminal part of nisin is then assumed to translocate across the membrane creating a pore in the target membrane. Thus, resulting in the inhibition of the target cells by either inhibition of cell wall synthesis or pore formation[452]. Figure taken from Rodney Honrada Perez *et al.*, 2015[453].

Lacticin 3147 is composed of two lanthionine-containing peptides, LtnA1 and LtnA2, with masses of 3,306 Da and 2,847 Da, respectively[454]. Lacticin 3147 requires both LtnA1 and LtnA2 to exert full antimicrobial activity. When both peptides are present, lacticin 3147 inhibits its target cells through the formation of pores in the cell membrane. Rapid efflux of K^+ and phosphate ions causes immediate dissipation of the membrane potential, hydrolysis of intracellular ATP and ultimately cell death[455]. The maturation of the subunits is performed by *LtnM1* and *LtnM2* genes and the bifunctional enzyme LtnT, which removes the leader peptide and translocates the modified peptide to the extracellular environment. Like Nisin A, Lacticin 3147 also targets lipid II, and a three-step model has been proposed where the α-peptide binds to lipid II, then it is recognized by the β-peptide which inserts into the membrane and forms a pore (Figure I.18)[455].

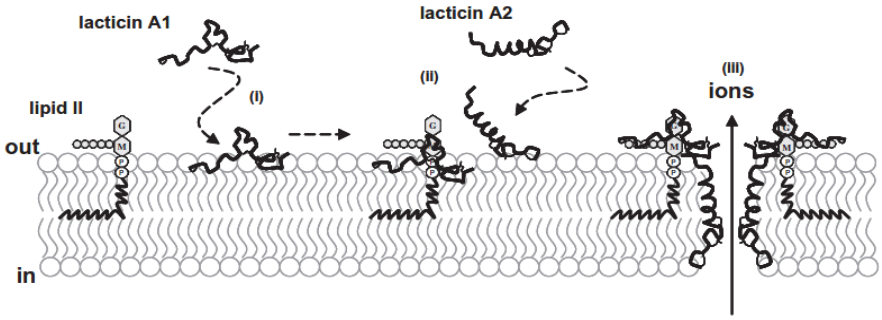


Figure I.18. Proposed model for Lacticin 3147 pore formation mechanism. First occurs the membrane association of the A1 peptide and binding to lipid II (i); Second there is a binding of the A2 peptide to A1:lipid II and formation of a high-affinity three-component complex (ii); Third there is a translocation of the C-terminus of A2 and formation of a defined pore in the membrane of the target (iii). Figure taken from Imke Wiedemann *et al.*, 2006[455].

Lactococcin G is a bacteriocin whose activity also depends on the complementary activity of two peptides, termed α and β, that can bind independently to the target cell surface[456]. Upon formation of a complex between the α and β peptides a transmembrane pore is formed, that

conducts monovalent cations (with the exception of protons)(Figure I.19)[456]. Thus, exposure of to lactococcin G results in rapid depletion of ATP and release of intracellular potassium[456].

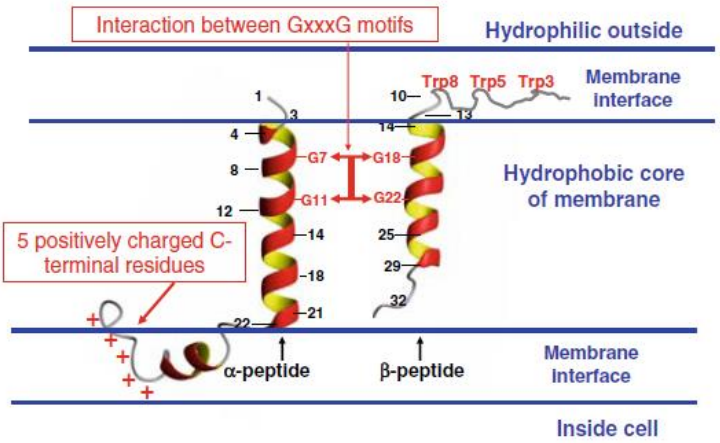


Figure I.19. The mechanism of interaction between Lactococcin G and the target membrane involves the binding of two peptides through specific motifs: G7xxxG11 in the α-peptide and G18xxxG22 in the β-peptide. These peptides come together to form a transmembrane helix–helix structure. The C-terminal end of the α-peptide, which is highly positively charged and structurally flexible, is driven through the membrane by the transmembrane potential. Meanwhile, the tryptophan residues located in the N-terminal region of the β-peptide, which is structurally flexible, are positioned either within or near the outer membrane interface. Figure taken from Jon Nissen-Meyer *et al.*, 2010[457].

Due to their antimicrobial properties, bacteriocins have been applied in various sectors ranging from food preservation to health. In this context, the use of bacteriocins to inhibit vaginal pathogens has been attempted. For example, in a review by Dover *et al.*, 2008, the authors compiled information regarding several bacteriocins that were isolated from *Lactobacillus* spp. and that shown efficacy in inhibiting growth of *N. gonorrhoeae*, *G. vaginalis* and *C. albicans* (as detailed in Table I.6)[458]. An important note is the fact that most studies that examined the antimicrobial effect of bacteriocins focused on lactobacilli producing species not endogenous to the vaginal tract.

Table I.6. Examples of studies demonstrating that bacteriocins have an inhibitory effect against potential bacterial vaginal pathogens (e.g., *G. vaginalis*) and fungal vaginal pathogens (e.g., *C. albicans*). The table describes the bacteriocin producing species, the name of the bacteriocin when available, the pathogen that it inhibits and the respective reference.

Producing species	Bacteriocin	Pathogen inhibited	Study
Bacterial pathogens			
***L. rhamnosus* 160**	Lactocin 160	*G.vaginalis*, *P. bivia*, *P. anaerobius*, *P. assacharoliticus*, *M. luteus*, *L. monocytogenes*	[443, 444, 459]
***Lactococcus lactis* subsp. lactis HV219**	Bacteriocin HV219	*E. faecalis*, *E. coli*, *L. casei*, *L. innocua, P. vulgaris*, *P. aeruginosa*	[460]
***L. fermentum* HV6b MTCC 10770**	Fermenticin HV6b	*G. vaginalis*	[461]
B. amyloliquefaciens	Subtilosin	*G. vaginalis*	[462]
***P. pentosaceus* SB83**	Bacteriocin SB83	*L. monocytogenes*	[463]
***E. faecium* strain 62-6**	Enterocin 62-6	*Corynebacteria*, *Streptococci*, and *Enterococci*	[464]
L. acidophilus	Bacteriocin with an expected size of 7.5 kDa	*G. vaginalis*, *S. agalactiae*, *P. aeruginosa*	[465]
***L. gasseri* EV1461**	Gassericin E	*S. aureus*; *S. intermedius*; *P. bivia*; *L. mesenteroides*; *G. vaginalis*; *E. faecium*	[466]
Fungal pathogens			
***L. pentosus* TV35b**	Pentocin TV35b	*C. albicans*	[467]
L. plantarum	Plantaricin peptides PlnE, -F, -J, and -K	*C. albicans*	[468]
E. faecalis	Bacteriocin EntV	*C. albicans*	[469]
E. durans	Durancin A5-11a and durancin A5-11b	*C. krusei*	[470]
***L. plantarum* WZD3**	Not characterized	*C. albicans*	[471]
L. pentosus, L. rhamnosus*, *L. paracasei* and *L. delbrueckii	Not characterized	*C. albicans and C. glabrata*	[472]

An important note concerning the bacteriocin-producing phenotype is that it is both strain and species dependent. For example, *L. gasseri* encodes acidocins (AAP55753.1 and AAP56345.1), gassericins (GaaA and GaeA) and helveticin J (encoded by *LGAS_RS02335*). However, in the genome of the reference strain only helveticin J protein is annotated and acidocins and gassericins are strain-specific, with the last being encoded in a plasmid that is not present in all strains[217-219]. A thorough search of the literature as well as inspection of the genomes of *L. gasseri*[466, 473-475], *L. reuteri*[230, 476], *L. rhamnosus*[459, 477, 478], *L. acidophilus*[383, 479, 480], *L. jensenii*, *L. iners*[481], *L. crispatus[482]*, *L. ruminis*[483], *L. plantarum*[484] and *L. salivarius*[485] were performed in order to render clear which bacteriocins are in fact (or could be) produced by these different species, this being something that was not

performed at a systematic level. The results obtained (schematically represented in Figure I.20) show that, with the exception of helveticin family proteins and gassericin A, which had homologs on the genomes of *L. gasseri*, *L. acidophilus* and *L. crispatus*, bacteriocins are quite species specific. *L. reuteri* LA6 strain was shown to produce the bacteriocin reutericin 6, its sequence had not been fully disclosed although it was found to be similar to the one of gassericin A[230]. Also, the sequences of lactocin 160[444] and rhamnosin A[478], produced by *L. rhamnosus*, and the sequence of inecin, produced by *L. iners*[481], were not determined and, thus, it was not possible to investigate their presence in the genomes of other lactobacilli species. *L. acidophilus*, on the other hand, encoded a helveticin family protein (WP_241234180.1), which had a great degree of homology with a helveticin encoded by *L. crispatus* (WP_158182237.1). Additionally, some strains of *L. acidophilus* also encoded a protein (AciA) similar to the gassericin A (GaaA), acidocin A (AcdA), acidocin J1132 (AJP47090.1), lactacin F (A37324) and lactacin B (AJP47084.1)[383]. *L. crispatus* encoded two different helveticin-like proteins, one similar to *L. gasseri* (WP_257942957.1) and the other similar to *L. acidophilus* (WP_158182237.1). *L. ruminis* was recently reported to produce a pediocin-like protein[483], and leucocin A (WP_129915276.1), with no evidence of these being present in the genome of other lactobacilli species. *L. plantarum* was previously demonstrated to produce a wide range of bacteriocins described as plantaricins (e.g., Plantaricin C, Plantaricin E/F and Plantaricin J/K, among others), that vary accordingly to the producing strain[484]. No homologues of these proteins could be found in the genome of other described lactobacilli species. Similarly, various *L. salivarius* strains produced various variations of salivaricin (e.g., salivaricin P, salivaricin L and salivaricin T, among others), which to our best knowledge were not produced by any of the remaining lactobacilli[485]. The vaginal species *L. jensenii* was not reported to produce any bacteriocins, nor we could detect in the available genome sequences homologs for those already characterized. In conclusion, most of these small peptides do not show a great degree of homology between species, which validates the observed structural differences among bacteriocin and their diversity. The fact that most of the bacteriocins produced by lactobacilli are poorly characterized highlights the need for their study since they can be a valuable option for bacterial and fungal infections treatment, including the development of new anti-*Candida* therapies.

Figure I.20. Selected bacteriocins produced by *L. gasseri*, *L. reuteri*, *L. rhamnosus*, *L. acidophilus*, *L. jensenii*, *L. iners*, *L. crispatus*, *L. ruminis*, *L. plantarum* and *L. salivarius*. Black square corresponds to the presence of the bacteriocin or a homolog in at least one strain of the corresponding species. This table was built using the available literature and primary amino acid sequence analysis (blastp) on NCBI (https://www.ncbi.nlm.nih.gov/). Bacteriocins with high degree of homology between species are represented by an asterisk (*1: *L. reuteri* - reutericin 6; *2: *L. acidophilus* - AciA; *3 and 4: *L. crispatus* – two helveticin-like proteins).

Bacteriocin	*L. gasseri*	*L. reuteri*	*L. rhamnosus*	*L. acidophilus*	*L. jensenii*	*L. iners*	*L. crispatus*	*L. ruminis*	*L. plantarum*	*L. salivarius*
Helveticin J							* 3			
Acidocin LF221A										
Acidocin LF221B										
Gassericin A				* 2						
Gassericin E		* 1								
Lactocin 160										
Rhamnosin A										
Helveticin							* 4			
Acidocin A										
Acidocin J1132										
Lactacin F										
Lactacin B										
Inecin										
Leucocin A										
Pediocin PA-1 family protein										
Plantaricin										
Salivaricin										

I.4.2.4. Production of hydrogen peroxide (H_2O_2)

Another metabolite produced by *Lactobacillus* spp. that has been hypothesized to contribute for its antimicrobial properties is hydrogen peroxide (H_2O_2). H_2O_2 is a toxic agent capable of inducing oxidative stress and, ultimately, cellular death in an array of microorganisms, including *E. coli*, *G. vaginalis* or *C. albicans*[220]. Production of H_2O_2 in the presence of oxygen is characteristic of most lactobacilli species[220]. Previous reports have shown that *L. acidophilus*, *L. gasseri* and *L. crispatus* produce, *in vitro*, between 100 and 800 μM H_2O_2 when grown aerobically[220]. Given the high production of H_2O_2 by lactobacilli, and the fact that vaginal microbiome studies consistently find significant reductions in levels of H_2O_2-producing *Lactobacillus* spp. during bacterial vaginosis[486, 487], the potential of H_2O_2 as an antimicrobial has been investigated. Some studies have assessed the role of H_2O_2 in restoring vaginal health, nonetheless, these studies showed that H_2O_2 was only effective in treating bacterial vaginosis and not infections caused by *Candida* species[488]. It is also important to denote that the amount of H_2O_2 in the vaginal fluid is about 25μM, substantially lower than the levels produced *in vitro*[489]. This difference could be attributed to the higher level of oxygen in the *in vitro* studies[220] and also to the H_2O_2-blocking activity of cervicovaginal fluid (CVF) that was shown to reduce H_2O_2 from the mili- to the micro-molar concentrations[489]. Importantly, studies have shown that *Candida* infections of the vaginal tract seem to be accompanied by a reduction of H_2O_2-producing *Lactobacillus* spp. (*L. crispatus* and *L. jensenii*) in infected patients[130, 140], suggesting clinical importance for H_2O_2 in VVC, although it is hard, if not impossible, to individualize this effect in the panoply of the other factors that can condition the anti-*Candida* activity of lactobacilli.

I.4.2.5. Production of biosurfactants

Surfactants produced by microorganisms, or biosurfactants, are amphipathic molecules containing a hydrophilic head (usually a peptide, amino acid, monosaccharide, disaccharide, or polysaccharide) and a hydrophobic tail with a linear, branched, saturated, unsaturated, or hydroxylated fatty acid[490, 491]. This combination of hydrophobic and hydrophilic moieties enables surfactants to reduce surface and interfacial tensions[490, 491]. Although lactic acid bacteria, including lactobacilli, have been reported to be good biosurfactant producers (well-reviewed by Surekha K. Satpute *et al.*, 2016[409]), the characterization of the production pathways and the environmental factors modulating this is much less studied than in other organisms that produce similar molecules (e.g. rhamnolipids produced in *P. aeruginosa* or surfactin produced by *B. subtilis*[492, 493]. The better characterized biosurfactant produced by different lactobacilli, including *L. acidophilus* where it was first isolated, is surlactin. This is a protein-rich cell associated biosurfactant released during the stationary phase and shown to reduce adhesion of *E. faecalis* to silicone rubber[494]. Like surlactin, the majority of the described biosurfactants produced by lactobacilli are cell-associated, with only a minor fraction being released into the medium[409]. The precise identification of the chemical composition of the biosurfactants produced by lactobacilli has also been not performed in most cases, being

described generalistic compositions in which the cell-free molecules are described to include a sugar and glycolipid-type moiety[495, 496], while cell-associated ones are described to have a proteinaceous portion along with glycoproteins, glycolipids and glycopeptides[409]. It is also worth mentioning that lactobacilli biosurfactant production, structural composition and activity are highly affected by the nutrients and conditions in the environment, such as protein abundance, micronutrients, pH and temperature[497], which further complicates the characterization of these molecules.

As said above, due to their chemical properties one of the more relevant aspects of the production of biosurfactants by lactobacilli is the reduction in the ability of other organisms to adhere to biotic surfaces. Indeed, it has been found that biosurfactants produced by *L. crispatus* vaginal isolates prevent the ability of *C. albicans*, *C. tropicalis* and *C. krusei* strains ability to adhere to human cervical epithelial cells, mainly by an exclusion mechanism[498]. Interestingly, delivery of this biosurfactant using hyalurosomes, reduced colonization of a broad spectrum of *Candida* species (*C. albicans*, *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, *C. krusei* and *C. parapsilosis*) onto a plastic surface[499]. Biosurfactants produced by *L. jensenii* P_{6A} and *L. gasseri* P_{65} showed inhibitory potential against *C. albicans*[221] as well the biosurfactants surfactin and iturin produced by vaginal strains of *L. plantarum*, *Latilactobacillus sakei* (formerly *Lactobacillus sakei*) and *L. fermentum,* that also showed to potently inhibit formation of *C. albicans* biofilms[500].

Table I.7. Selected examples of biosurfactants produced by *Lactobacillus* spp. The table describes the species and strain of the producer, when available a brief description and the antimicrobial/antifungal potential of the biosurfactant.

Species	Biosurfactant description	Main outcome	Study
***L. casei* subsp., rhamnosus 36**	Surlactin	Protein rich biosurfactant that may interfere with uropathogen adhesion (e.g., *E. faecalis*)	[501]
***L. brevis* CV8LAC**	Biosurfactant with a mixture of components including sugar as one of the fractions	Inhibition of adhesion, biofilm formation of *C. albicans*	[495]
***L. reuteri* DSM 20016**	Not described	Inhibition of *S. mutans* initial adhesion to the tooth surface	[234]
L. acidophilus	Surlactin	Not tested as antimicrobial or antifungal	[497]
Lactobacillus* vaginal isolates, including *L. plantarum*, *L. sakei* and *L. fermentum	Surfactin and iturin	Anti-adhesive activity (up to 74.4% reduction of the biofilm formed in abiotic surface) against *C. albicans*.	[500]
***L. acidophilus* RC14**	Surlactin	Inhibit *E. faecalis* adhesion to silicone rubber	[502]
***L. jensenii* P_{6A} and *L. gasseri* P_{65}**	Biosurfactant was composed of 51.49 to 38.61% carbohydrate, 15.17 to 9.81% protein and 29.45 to 49.53% lipids.	Antimicrobial activity against clinical isolates of *E. coli*, *S. saprophyticus*, *E. aerogenes*, *K. pneumoniae* and *C. albicans.*	[221]

I.4.2.6. Production of exopolysaccharides

Exopolysaccharides (EPSs) are large carbohydrates with diverse chemical properties and functions that are intimately related to their structure and origin. Capsular exopolysaccharides (cEPSs) form a capsule around cells, while slime exopolysaccharides (sEPSs) are released into the surrounding environment. EPSs can be classified into two groups based on their monomer composition: homopolysaccharides, composed of a single type of monomer, and heteropolysaccharides, composed of two or more types of monomers. Additionally, these can be linear or branched chained, with the bonds between the monomers being β-1-3, β-1-4, α-1-2 or α-1-6[503]. EPSs have multiple functions including the enhancement of surface adhesion, facilitation of biofilm formation and protection against environmental aggressions[503]. Lactobacilli EPSs are characterized by their exceptional biocompatibility, high viscosity, and ability to stabilize emulsions[503]. Consequently, in the food industry, these EPSs are utilized as bio-thickeners due to their emulsifying properties. EPSs produced by *Lactobacillus* species are both homopolymeric and heteropolymeric. The gene organization and functional properties of the EPSs gene clusters have been studied in a few lactobacilli species, including *L. gasseri*, *L. reuteri*, *L. delbreuckii*, *L. helveticus*, *L. johnsonii*, *L. paraplantarum*, *L. plantarum* and *L. rhamnosus*[242]. Such analyses revealed a great diversity among the EPS gene clusters (for example in the number of copies in the genome) being in common the presence of regulatory genes on the 5'end; polymerization- and export-related genes on the 3'end; and glycosyltransferase (gt) genes at the centre of the cluster[504]. The generic EPS gene cluster organization include a transcriptional regulator, *epsA*; a phosphoregulatory module, *epsBCD*; and a priming glycosyltransferase, *epsE* arranged in a tandem array commonly called *epsABCDE*[504]. In *Lactobacillus*, it appears that EPS gene clusters comprise a glycosyltransferase (*epsE*), a glycosyltransferase (*gt*), a flippase (*wzx*), a polysaccharide polymerase (*wzy*), a tyrosine kinase (*epsC*) and a tyrosine kinase modulator (*epsB*)[242]. Other genes which are often present as a part of some of the EPS clusters of lactobacilli include LytR transcriptional regulator (*epsA*), phosphotyrosine phosphatase (*epsD*), involved in the generation of activated sugar precursors and acetyl- and pyruvyl transferases, involved in the chemical decoration of the EPS[242]. An extensive analysis of the EPSs gene cluster of 106 *Lactobacillus* strains reported that most strains had an organization similar to the abovementioned, although some clusters appear incomplete by lacking the polysaccharide polymerase (*wzy*), the phosphoregulatory module (*epsB* and *C* or both), the flippase (*wzx*) or the priming glycosyltransferase (*epsE*)[242]. Such comparative analysis also point to EPS production being a phenotype that is highly strain-dependent[242]. Studies have identified the EPS gene cluster of some species that are commonly found in the human host including in *L. gasseri*, *L. crispatus*, *L. reuteri*, *L. acidophilus*, *L. plantarum*, *L. rhamnosus and L. salivarius*, showing that although consisting of the elements described above, they had very distinct cluster organization with some cluster having more or less of these genes (e.g., different numbers of glycosyltransferases and transposases) (Figure I.21)[242, 505].

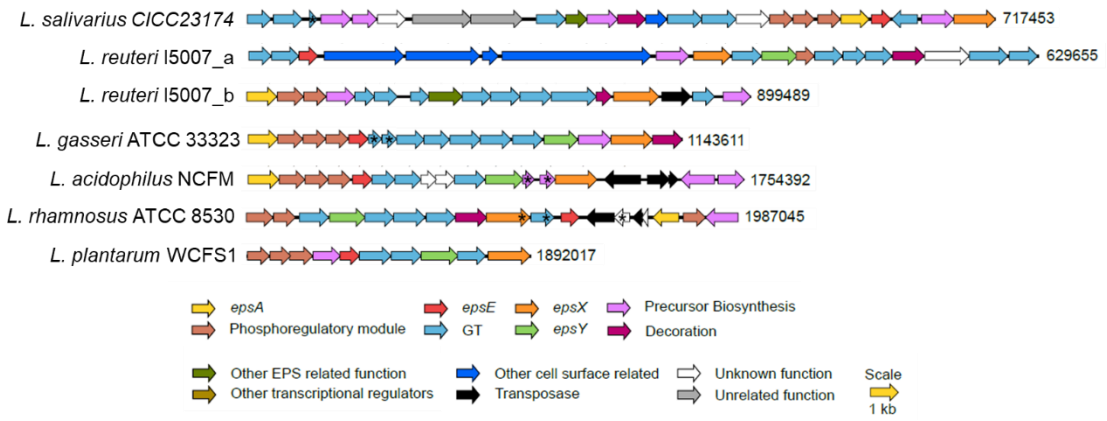


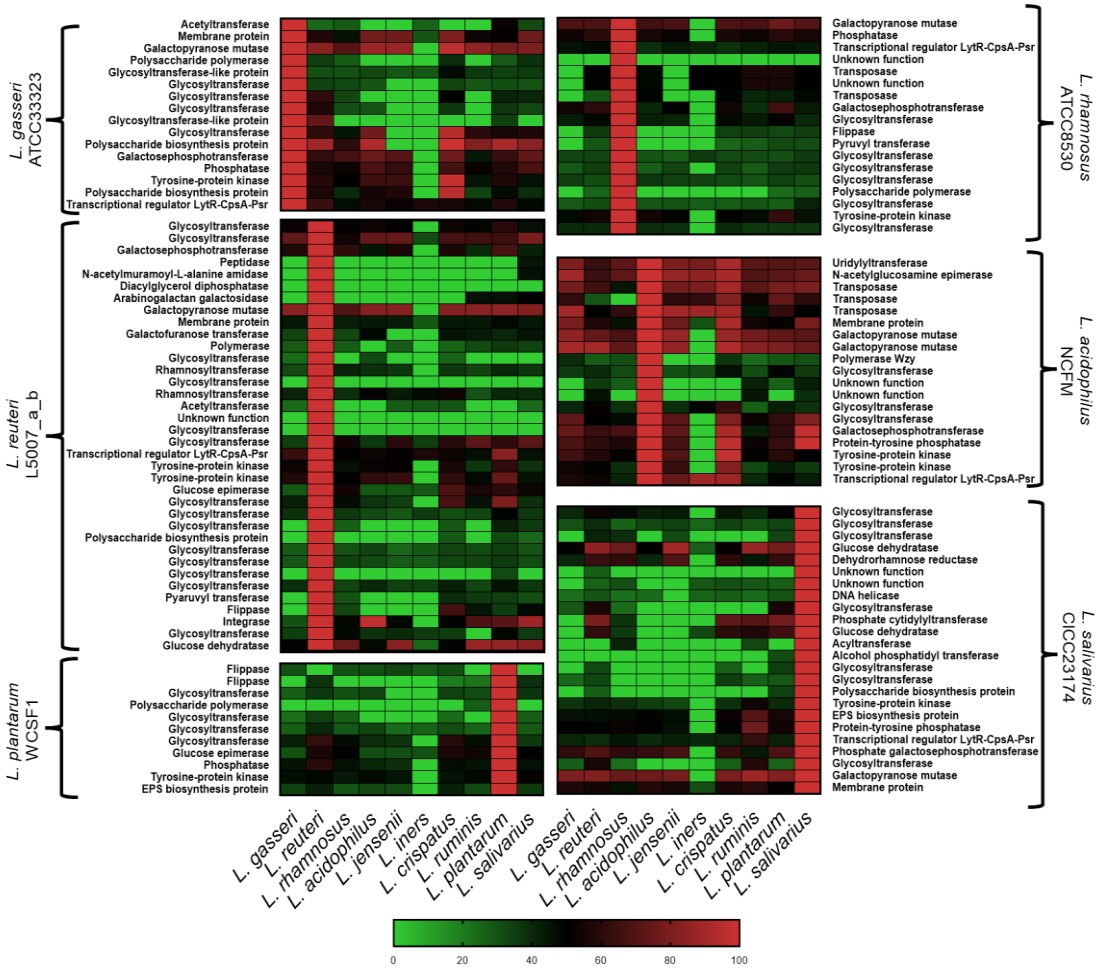
Figure I.21. EPS gene cluster organization of *L. reuteri* (two clusters), *L. gasseri*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *L. salivarius* strains selected from the analysed cohort of Dipti Deo *et al.*, 2019[242]. Note that gene clusters are composed of different genes, number of genes, and the overall organization of the cluster is completely different, which could originate distinct EPSs. The number in front of the cluster indicates the cluster genomic location. Adapted from Dipti Deo *et al.*, 2019[242].

The antimicrobial and antifungal properties of lactobacilli EPSs has been addressed, with far less descriptions for the antifungal applications. Indeed, EPS produced by *L. plantarum*, *L. gasseri*, *L. casei* and *L. rhamnosus* was found to inhibit growth of *E. coli*, *E faecalis* and *S. aureus* as reviewed in Abdelmoneim K. Abdalla *et al.* 2021[506]. One study isolated a cohort of *Lactobacillus* strains (5 *L. brevis*, 2 *L. pentosus*, and 7 *L. plantarum*) from traditional fermenting green olives and demonstrated that their EPSs exhibited antifungal properties against *C. pelliculosa*[507], albeit in a strain-dependent manner. Additionally, *L. plantarum* R315 was also shown to produce an EPS with inhibitory activity against *C. albicans*[410] while the EPS produced by *L. rhamnosus* GG reduced the ability of *C. albicans* to form hyphae and adhere to vaginal epithelial cells[508].

A blastp analysis of the proteins described as being part of the EPS gene clusters of the vaginal (*L. gasseri*), intestinal (*L. reuteri* and *L. salivarius*) and other species also found in the human host (*L. rhamnosus*, *L. acidophilus*, and *L. plantarum*) was performed. The proteins belonging to the EPS cluster of the above species were compared with the proteome of each other and with three other vaginal species (*L. crispatus*, *L. iners* and *L. jensenii*) and one other intestinal species (*L. ruminis*). This comparative analysis rendered clear that the proteins belonging to the EPS clusters are represented in the genome of all the lactobacilli species examined, although with different degree of homologies, suggesting that they are all EPS producers (Figure I.22 panel A). Among the EPS proteins, UDP-galactopyranose mutase was the most conserved protein of the clusters. Despite this, significant variability was observed in the amino acids sequences of the different components. For example, the proteins of the EPS cluster belonging to *L. rhamnosus* ATCC 8530 and *L. plantarum* WCSF1 were significantly different to their corresponding orthologues in the remaining species used in this comparative analysis (Figure I.22 panel A). Another observation of remark was that the *L. salivarius* EPS cluster seems to be comprised of proteins very dissimilar of those encoded by the other species with the exception of dTDP-glucose 4,6-dehydratase and UDP-galactopyranose mutase that were similar to those found in the counter-partner species.

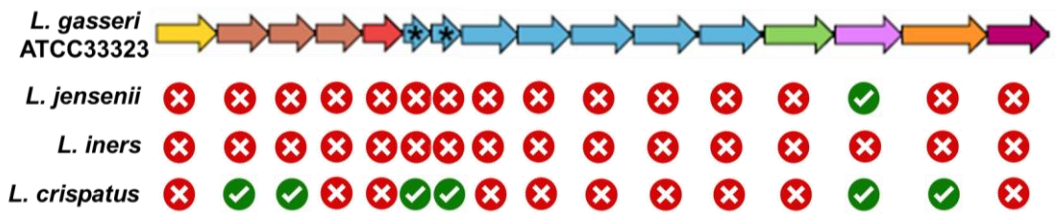
For a better visualization we have constructed the representation in Figure I.22 panel B for *L. gasseri* and *L. reuteri*. Comparing the EPS cluster of the vaginal species *L. gasseri* and intestinal species *L. reuteri* revealed that their EPS gene cluster is unique and with very little homology with other vaginal or intestinal species. For example, only *L. crispatus* showed some conservation of the proteins responsible for EPS production of *L. gasseri*, while in the intestinal species only a galactopyranose mutase and a glucose dehydratase were also encoded by other lactobacilli. It is interesting that among all of the proteins of the EPS clusters from various species, the galactopyranose mutase appear to be conserved among all lactobacilli, suggesting a feature that could be crucial and thus present in most lactobacilli genomes.

A



B

***L. gasseri* (vaginal species)**



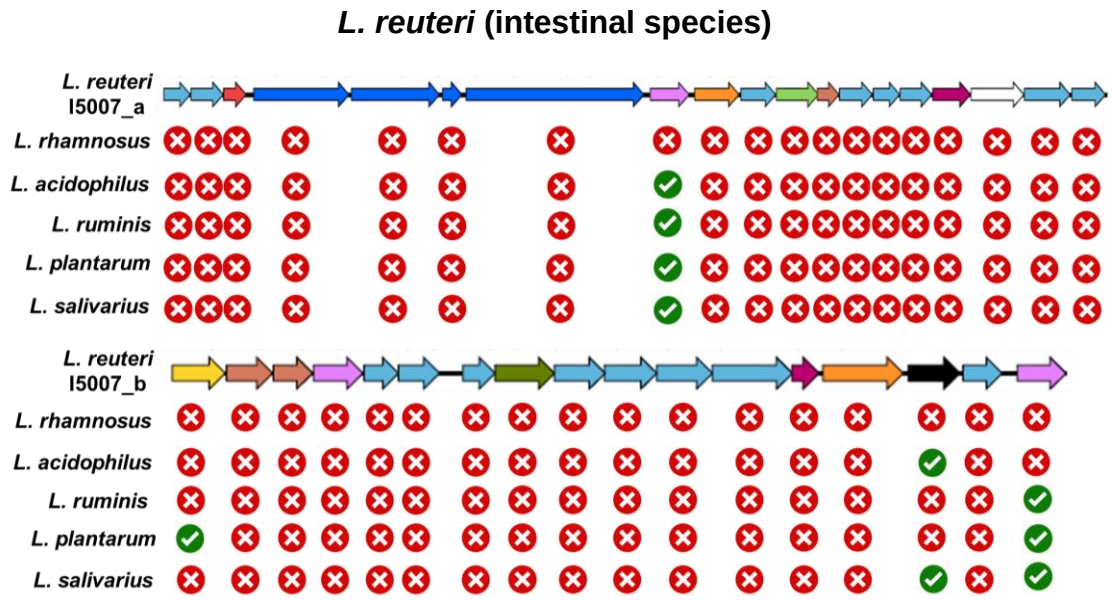


Figure I.22. (A) Heat map representing the similarity between the EPS gene clusters of the strains *L. gasseri* ATCC 33323, *L. reuteri* ZLR003 *L. reuteri* I5007, *L. rhamnosus* ATCC 8530, *L. acidophilus* NCFM, *L. plantarum* WCSF1 and *L. salivarius* CICC23174 with the EPS genes present in the representative genomes of *L. gasseri*, *L. reuteri*, *L. acidophilus*, *L. jensenii*, *L. iners*, *L. crispatus*, *L. ruminis*, *L. plantarum* and *L. salivarius* deposited in the NCBI. Colour represents the identity of the best-hit protein in the representative genome of the species in the x-axis. Low identity proteins are in green while similar proteins are in red. **(B)** Schematic representation of the presence of the EPS cluster proteins of *L. gasseri* and *L. reuteri* in the EPS cluster of the remaining species. Cross indicates no protein homologous while green check mark indicates proteins with high degree of homology. Proteins were considered highly similar when their identity was above 80% and with a p-value under 10^{-20} accordingly to the blastp results. Protein sequences were obtained from the work of Dipti Deo *et al.*, 2019[242].

I.4.2.7. Immunomodulation

The effects of lactobacilli on innate immune and adaptative responses have been shown to be exerted via their binding to pattern recognition receptors (PRR), such as toll-like receptors (TLR), expressed on immune cells and many other tissues such as the intestinal and vaginal epithelium[509]. These studies, reviewed and compiled by Atieh Abedin-Do *et al.* 2015, have been reporting that the presence of lactobacilli, including *L. crispatus*, *L. gasseri*, *L. reuteri*, *L. johnsonii* and *L. acidophilus*, among others, is able to induce significant changes in the immune system of the host[509]. In particular, the presence of this bacteria increased expression of the pro-inflammatory cytokine INF-γ and modulated the expression of defensin proteins, involved in host immunity[509]. In vaginal cells infected with *C. trachomatis*, the presence of *L. crispatus* reduced the production of the pro-inflammatory cytokines IL-6 and IL-8 and TNF-α and increased the production of IL-10, anti-inflammatory[510]. The presence of *L. crispatus* was also reported to reduced production of pro-inflammatory interleukin 8 and human beta defensin 2 while it increased expression of beta defensin 3 (HBD-3) along infection of vaginal epithelial cells with *C. albicans*[511]. The authors additionally concluded that HBD-3 was effective in killing *C. albicans* cells, proposing that *L. crispatus* also protects the epithelial cells against *C. albicans* virulence by inducing the expression of HBD-3[511]. Importantly, lactic acid and H_2O_2, produced by lactobacilli, have also been suggested to serve as important immunomodulators and above it has already

been described the important role of lactate as an inducer of immune evasion prompted by *Candida* cells. Moreover, lactate was also able to induce an anti-inflammatory response prompted by vaginal cells by stimulating the production of the anti-inflammatory cytokine IL-1RA as well as suppressing the production of pro-inflammatory cytokines including IL-6 and IL-8 and TNFα[512]. Additionally, the presence of H_2O_2 producing lactobacilli was also found to reduce the production of pro-inflammatory cytokines IL1β, leukocyte protease inhibitor and of beta defensin 2 (HBD-2)[513]. It is worth mentioning that the immune modulation prompted by the presence of *L. crispatus* has similar features to the immunomodulatory effects observed for lactate (e.g., both reduced expression of pro-inflammatory cytokines IL-6 and IL-8).

I.4.2.8. Global responses of *Candida* to the presence of lactobacilli

The majority of the studies addressing the anti-*Candida* potential of lactobacilli are based on phenotypic observations in which it is concluded about the effect of the presence of a specific species or strain in inhibiting growth and/or virulence traits of the pathogenic yeasts, with little to no explanation of the molecular mechanisms that underly such antagonism. More recently, a few studies paved the way into a better understanding of how *Lactobacillus* and *Candida* respond to each other at a more molecular level and, in particular, at a transcriptome-wide scale. Alonso-Roman *et al.* 2022 focused on the interaction established between *L. rhamnosus* and *C. albicans* and for that the authors undertook a transcriptomic and metabolomic profiling of a tri-culture containing *C. albicans*, *L. rhamnosus* and intestinal epithelial cells (IECs). Interestingly, the medium (KBM) used for cultivation intestinal epithelial cells was not sufficient to sustain the growth of *L. rhamnosus*, however, when this medium was supplemented with an intestinal epithelial cell culture supernatant growth of the bacterium was observed[398]. This observation strongly suggests that epithelial-secreted metabolites foster bacterial growth with the authors attributing this to the excretion of carnitine, citric acid and gamma-glutamylalanine[398]. Another important finding was that *L. rhamnosus* was only able to suppresses *C. albicans* pathogenicity against human intestinal epithelial cells, when in a metabolically active state[398]. This observation fostered a subsequent metabolomic analysis of a *L.* rhamnosus:intestinal epithelial cells culture supernatant, that was found to be enriched in intermediates of pyrimidine metabolism; pantothenate and CoA biosynthesis; phenylalanine, tyrosine and tryptophan biosynthesis; beta alanine metabolism and thiamine metabolism[398]. Among the metabolites that were only found to be secreted by *L. rhamnosus* in the presence of the intestinal cells, the authors identified phenyllactic acid, mevalonolactone, 2-hydroxyisocaproic acid (HICA), and 3-hydroxyoctanoate. Interestingly, these molecules were before shown to have some antifungal potential, including against *Candida*[399, 401-403, 405]. Moreover, the authors reported that *L. rhamnosus* produced hydroxyphenyllactate, DL-indole-3-lactic acid, 2-hydroxyisocaproic acid and α-hydroxicaproic acid, that favoured the hyphal-to-yeast transition[398]. Similarly, *L. rhamnosus* was also shown to deplete glucose, fructose, mannose, N-acetylglucosamine/N-acetylgalactosamine, serine, phenylalanine and leucine from the medium, these being major carbon and nitrogen sources supporting growth of *C. albicans*[398]. Consistent with these modifications in the environment,

transcriptomics analysis of *C. albicans* reflected a broad metabolic adaptation characterized by down-regulation of glycolysis-relevant genes and oxidative phosphorylation pathway and an up-regulation of several TCA cycle and glyoxylate shunt genes (Figure I.23)[398].

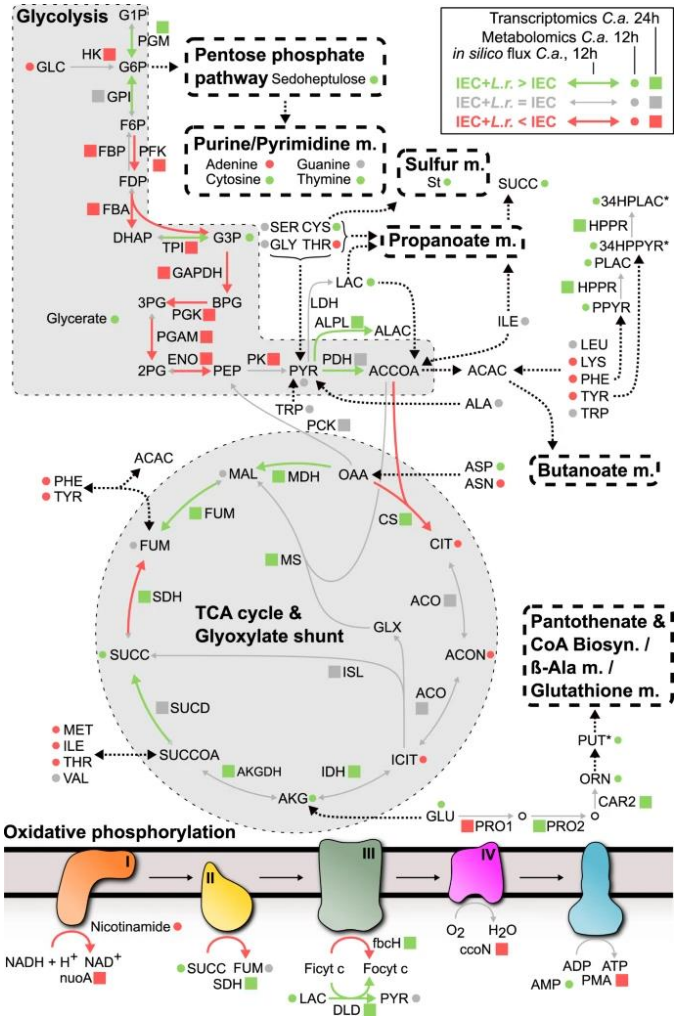


Figure I.23. Model proposed by Alonso-Roman *et al.*, 2022 of the survival adaptations of *C. albicans* in the presence of *L. rhamnosus* during intestinal epithelial cells (IECs) infection. Data was gathered from *in silico*, metabolomics, and transcriptomic approaches. Green pathways indicate increased expression and red pathways indicate decreased expression, while grey pathways were not significantly changed in the presence of *L. rhamnosus*. Figure taken from Raquel Alonso-Roman *et al.*, 2022[398].

The interaction of *C. albicans* with *L. crispatus* was also inspected at a more molecular level by McKloud *et al.*, 2021, however, in this case focusing the transcriptomic analysis observed in a mixed biofilm formed by these two species. Notably, in the early time points of 6 and 8h, gene expression of *C. albicans* was not significantly different in the mixed-biofilm than in the single-species biofilm[140]. At 24h, however, the situation was different and it was found that the presence of *L. crispatus* induced the up-regulation of *C. albicans* genes involved in amino acid biosynthesis and breakdown (e.g. *ARG3*, *BAT21*, *ILV1*, and *HIS5*), in vitamin metabolism and in α-amino acid biosynthesis (Figure I.24)[140]. An important down-regulation of the *C. albicans PRY1* gene was also observed this being of relevance due to the important role of this protein in mediating virulence of this yeast[140]. On the overall, the authors also concluded that the presence of *L. crispatus* modified the nutritional portfolio of the medium, causing *C. albicans* to

enter a starvation state[140], this having some analogy with the modifications that had also been observed in the *C. albicans-L. rhamnosus* interaction described above.

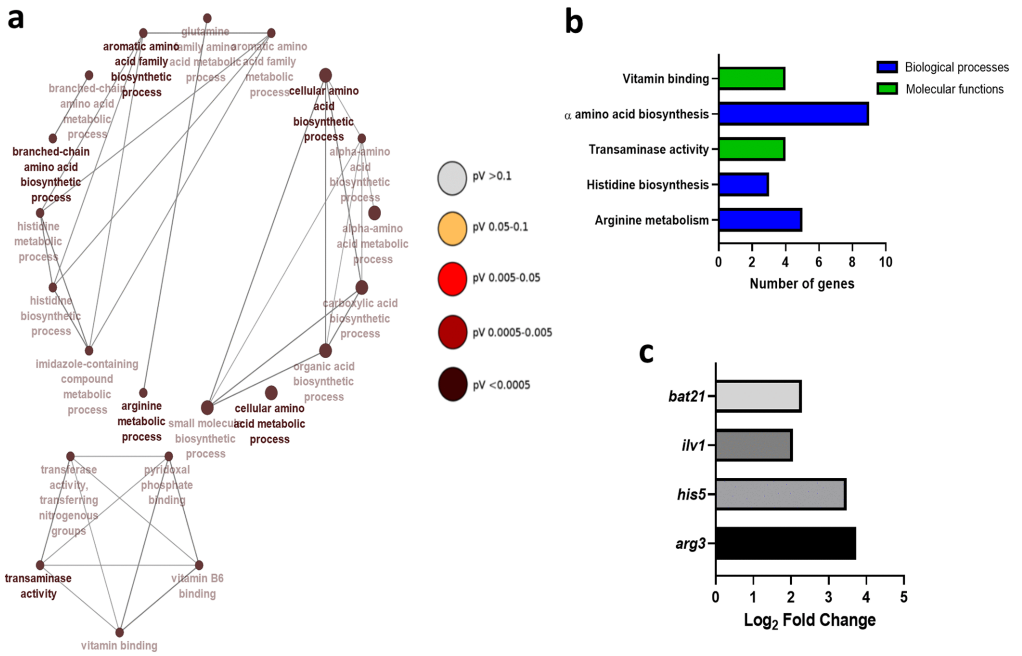


Figure I.24. Gene networks of gene ontology (GO) terms and up-regulated genes in dual-species biofilms of *C. albicans* and *L. crispatus*. **(A)** GO terms of *C. albicans* genes differentially regulated after 24h; **(B)** Biological functions of up-regulated genes of *C. albicans* in dual-species biofilms considered relevant for adaptation to *L. crispatus*; **(C)** Log_2 fold change of genes identified by the authors to be important for the growth of *C. albicans* in the presence of *L. crispatus*. Figure taken from McKloud *et al.*, 2021[140].

I.5. Thesis aims

Historically, lactobacilli have been associated with improved benefits in human health, in part, by protecting against infection caused by pathogens. Although studies have demonstrated that indeed commensal lactobacilli microflora abundance provides protection against bacterial infections of the vaginal mucosa, such protective effect against infections caused by pathogenic yeasts of the *Candida* genus (also frequent vaginal pathogens) is still elusive. Despite this, several studies, either involving clinical trials resorting to probiotic cocktails based on lactobacilli species or *in vitro* analyses of the inhibitory effect of different lactobacilli species/strains, support this idea that it could be possible to use lactobacilli to enhance protection against *Candida*. It is in this context that emerged this thesis, embedded in the activities of the research project LactoCan that focused on the investigation of the interactions established between *Candida* species and indigenous lactobacilli species of the vaginal and intestinal tract, that are, in most cases, still very poorly explored. In such context, it were specific objectives of this thesis:

i) to evaluate the anti-*Candida* potential of the vaginal species *L. gasseri* along cultivation, in planktonic or in biofilm-forming conditions, and the environmental parameters that might modulate this antagonism. For that, experimental settings allowing monitoring of cellular viability of *C. albicans*, *C. glabrata* and *L. gasseri* while growing in the presence of each other were

implemented. Different from most work undertaken until thus far, in this thesis we have explored direct co-cultivation of the different species instead of using bacterial supernatants as proxies.

ii) investigate the *L. gasseri-Candida* interaction at molecular level gathering knowledge on the genes and pathways determining the response of these species to the presence of each other as well as the genes that assure growth in those co-cultures. For that, transcriptomic analyses (based on dual RNA-sequencing) coupled with large-scale phenotypic screenings were conducted in co-cultures established between *C. glabrata* and *L. gasseri* along planktonic growth and in mixed biofilms. While the identification of genes improving *L. gasseri* anti-*Candida* potential is relevant to design what can be better performing strains, the identification of *Candida* genes assuring survival of these yeasts in the presence of the bacteria provides a novel cohort of therapeutic target genes.

iii) investigate differences and similarities between the antagonistic potential of *L. gasseri* and the one of the intestinal species *L. reuteri*. Although candidiasis is a well-known pathology of the vaginal tract, research is showing that resident *Candida* commensal populations in the gut are able to invade the bloodstream causing life-threatening infections. In the gut, like in the vaginal tract, there is also lactobacilli species and thus the same approach of developing probiotic-based approaches to control the commensal *Candida* populations in the gut may also be useful. In this context, the experimental setting previously established for *L. gasseri* was explored to investigate how *L. reuteri* interact with *C. albicans* and *C. glabrata*.

The following chapters describe the findings that were obtained to pursue these objectives, followed by a comprehensive discussion that aims to integrate the state-of-the-art described in the introduction while putting in context the results obtained in this thesis.

II. Acetate modulates the inhibitory effect of *Lactobacillus gasseri* against the pathogenic yeasts *Candida albicans* and *Candida glabrata*

Part of the results shown in this chapter were published in:

Pedro, N. A., Fontebasso, G., Pinto, S. N., Alves, M., & Mira, N. P. “Acetate modulates the inhibitory effect of *Lactobacillus gasseri* against the pathogenic yeasts *Candida albicans* and *Candida glabrata*.” **Microbial Cell**, 10(4), 88, 2023.

II.1. Abstract

The exploration of the interference prompted by commensal bacteria over fungal pathogens is an interesting alternative to develop new therapies. This chapter scrutinized how the presence of the poorly studied vaginal species *L. gasseri* affects relevant pathophysiological traits of *C. albicans* and *C. glabrata*. *L. gasseri* was found to form mixed biofilms with *C. albicans* and *C. glabrata* that involve close cell-cell contacts that cause a pronounced death of the yeast cells, while bacterial viability is not affected. Reduced viability of the two yeasts was also observed upon co-cultivation with *L. gasseri* under planktonic conditions. Either in planktonic cultures or in biofilms, the anti-*Candida* effect of *L. gasseri* was augmented by acetate, in a concentration-dependent manner. The two *Candida* species also counteracted the acidification prompted by *L. gasseri* impacting the balance between dissociated and undissociated organic acids. Importantly, this feature could not be phenocopied in single-cultures of *L. gasseri* (that accumulated high amounts of acetic acid, while in the co-culture prevailed the non-toxic acetate). Altogether these results herein described are expected to advance the design of new anti-*Candida* therapies based on probiotics, in particular, those based on vaginal lactobacilli species helping to reduce the significant burden that infections caused by *Candida* have today on human health.

II.2. Introduction

Candidiasis is an infection caused by pathogenic yeasts of the *Candida* genus and account for around 50 to 70% of the reported fungal infections worldwide[514]. Infections caused by *Candida* are more frequent in the oral or in the vaginal mucosa, but in more serious cases, often life-threatening, these yeasts disseminate in the bloodstream and colonize major organs[268, 515]. Due to their high mortality rates, aggressiveness and recurrence, infections caused by *Candida* have a very high societal impact[516]. The occurrence of systemic candidiasis is most frequently observed upon immunosuppression (e.g., in patients undergoing chemotherapy or in the elderly), but vaginal candidiasis is common among the healthy female population. Women are estimated to suffer 2-3 episodes of vaginal candidiasis during their lifetime with a significant proportion (5 to 10%) suffering from recurrent infections, a condition known as recurrent vulvovaginal candidiasis (RVVC)[250, 517]. The more relevant species causative of candidiasis, both superficial and systemic, is *Candida albicans* but the incidence of infections caused by non-albicans *Candida* species (also called as NACS) is increasing, in some geographies prominently[255, 256]. Infections caused by NACS are worrisome as these species are usually very resilient to commonly used antifungals and the underlying infections have poorer outcomes for patients, compared to infections caused by *C. albicans*[257, 258]. Among NACS *Candida glabrata* is usually the more prevalent species, in part due to its innately high tolerance to azoles and extreme genomic plasticity that, among other traits, prompts fast adaptive responses to the challenging environment of infection sites[257, 259].

A distinguishing aspect of the pathophysiology of *Candida*, compared with other human-infecting Fungi, is that these species are present as part of the microbiota of various sites, even in the absence of disease[75, 100, 518]. Indeed, *C. albicans* has been identified as a true gut symbiont based on its consistent identification in resident microbial populations of this niche[75, 100]. *C. glabrata* has also been identified in the gut microbiome, however not consistently, remaining to be elucidated whether it is a true symbiont or a transient passenger[248, 519]. *C. albicans* and *C. glabrata* have also been identified in the vaginal mycobiome of “healthy” women, using both culture-dependent and independent methods[101-103]. However, these yeasts are not ubiquitously observed in all samples suggesting that the presumed “healthy” women could be asymptomatic carriers and thereby leaving open whether or not the vaginal tract is a primary site of colonization for these two *Candida* species. While in the past not much attention was given to these commensal populations of *Candida*, in the recent years it has been demonstrated that they can be reservoirs for dissemination, especially in the gastrointestinal tract[100, 268].

Besides *Candida* a plethora of other species compose the vaginal microbiome, their identity and abundance differing with the anatomic site (prominent differences were observed between the cervix or the uterus), with age, habits or race[520-522]. Despite the inter-individual variation, it is clear that the vaginal microbiome is dominated by lactobacilli with *L. iners*, *L. crispatus*, *L. jensenii* and *L. gasseri* being the most abundant[96, 97]. Perturbation of the vaginal microbiome was linked to adverse gynaecologic/obstetric outcomes including preterm birth[50],

mucosal inflammation[523] or infections caused by HPV[524], HIV[525] or bacteria[522, 526]. Studies examining the vaginal microbiome of asymptomatic women or of patients with diagnosed vaginal candidiasis obtained conflicting results, with some reporting decreased abundance of the lactobacilli population, others reporting changes in the species profile (for example, an unusual predominance of *L. iners*) and others reporting no alterations in the habitual lactobacilli-enriched flora[102, 130, 136-140, 527, 528]. Although it is not totally clear whether vaginal lactobacilli provide protection against candidiasis *in vivo*, it has been well reported the potential of those species in inhibiting growth and relevant pathogenic traits of *Candida in vitro*[390, 394, 529]. These observations opened the door to the use of probiotics based on lactobacilli as possible anti-*Candida* treatments with results pointing to a positive contribution to the prevention of relapse and avoidance of recolonization[377, 530, 531]. However, most of these probiotic cocktails were developed using lactobacilli species not indigenous to the vaginal tract, likely due to the poor knowledge available concerning their genetics and physiology[530, 532]. Intestinal lactobacilli species (which differ from those found in the vaginal tract) have also been found to restrain the growth and virulence of *Candida in vitro* and in infection models[398, 533, 534].

Resulting from this interest in the exploitation of the lactobacilli microflora as potential anti-*Candida* agents some studies examined this interference in more detail (as reviewed in Graf *et al.*, 2019, Zangl *et al.*, 2019 and d'Enfert *et al.*, 2021)[85, 534, 535]). Usually, the role of lactobacilli as drivers of vaginal health was attributed to lactic acid production which maintains an acidic vaginal pH and restrains bacterial growth[411, 536, 537]. However, the acidophilic nature of yeasts, including *Candida* spp, along with the demonstration that at pH 4 (the usual pH of a vaginal fluid dominated by lactobacilli[538]), physiologically relevant concentrations of lactic acid don't inhibit the growth of *C. albicans* or *C. glabrata*[430], suggests that lactic acid may play a minor role in restraining *Candida*. Interestingly, it has been shown that lactate promotes the evasion of *Candida* cells from the immune system by inducing β-glucan masking and reducing macrophage recruitment[289], suggesting that these yeasts evolved adaptive responses to allow them to cope with the presence of this acid anion in the environment in a manner that favours colonization and, eventually, infection. Additionally, lactic acid was shown to be a potent immunomodulator, reducing the production of inflammatory cytokines by epithelial cells thereby containing inflammatory responses[406, 512, 539]. More recently, the anti-*Candida* potential of some lactobacilli species was attributed to the production of 1-acetyl-β-carboline[395], however, the only vaginal lactobacilli species studied was *L. gasseri* and the authors concluded that under their experimental setting, supernatants obtained from culturing this bacterium inhibited filamentation of *C. albicans*, but not growth[395]. Other studies also demonstrated reduced filamentation and growth of *C. albicans* when cultivated in medium supplemented with supernatants obtained from *L. gasseri*, *L. jensenii* or *L. crispatus* cultures, although strain-to-strain variation was observed, especially for the *L. gasseri* strains[394]. Other described anti-*Candida* effects attributed to lactobacilli involve the reduction in the ability of these yeasts to bind to epithelial cells due to a higher affinity for epithelial receptors and secretion of biosurfactants[387, 389, 495, 500] or the production of bacteriocins, although no identification of relevant players had

been identified[468, 472]. However, no specific molecular players mediating these responses have been disclosed.

In this chapter, was examined the growth, physiology and virulence traits of *C. albicans* and *C. glabrata* when co-cultivated with the poorly studied vaginal species *L. gasseri* under planktonic or biofilm-forming conditions. This combined approach is a distinct aspect of our work since most studies addressing this interaction used the cultivation of the yeasts in the presence of supernatants produced by vaginal bacterial cultures and not directly the cell-cell interaction. Besides assessing relevant physiological aspects of the interaction established between these two species, it was also uncovered the role of acetate as a positive modulator of the interference of *L. gasseri* over *Candida* even in concentrations similar to those found in a vaginal microflora dominated by lactobacilli.

II.3. Materials and methods

II.3.1. Strains and growth media

In this chapter were used the reference strains *L. gasseri* ATCC 33323 (acquired from DSMZ); *C. glabrata* KUE100 (a wild-type strain derived from the CBS138 strain[433]); and *Candida albicans* SC5314. We have also made use of five clinical strains: *L. gasseri*, ISTLg97, a vaginal isolate whose species identity was confirmed by MALDI-TOF and based on sequencing of the 16S RNA sequence; *C. glabrata* VG49, *C. glabrata* VG216, *C. albicans* VG217 and *C. albicans* VG485, all vaginal strains that had been recovered along epidemiological surveys undertaken in the Lisbon area[540]. The MRS medium used to co-cultivate yeasts and bacteria contains, per litre, 10 g casein peptone (Gibco); 10 g meat extract (Panreac AppliChem); 5 g yeast extract (Gibco); 20 g glucose (Nzytech); 1 g Tween 80 (Sigma); 2 g K_2HPO_4 (Merck); 5 g sodium acetate (Merck); 3 g ammonium sulphate (Panreac AppliChem); 0.20 g $MgSO_4.7H_2O$ (Labchem) and 0.05 g $MnSO_4.H_2O$ (Sigma). After preparation, the pH of MRS was adjusted to 6.2-6.5 using HCl or NaOH. In indicated experiments, the sodium acetate used to prepare MRS was replaced by sodium chloride (Honeywell, Fluka™). YPD medium, used for maintenance of the strains, contains, per litre, 20 g glucose (Nzytech), 20 g peptone (Gibco) and 10 g yeast extract (Gibco). Solid YPD or MRS were prepared by supplementing the corresponding liquid medium with 2% and 1.5% agar (Nzytech), respectively. Media were prepared using deionized water and sterilized by autoclaving for 15 min at 121°C and 1 atm.

II.3.2. Co-cultivation in liquid MRS medium of *L. gasseri* with *C. glabrata* or *C. albicans*

To examine the growth of *L. gasseri* in liquid MRS, alone or in the presence of *C. glabrata* or *C. albicans*, a pre-inoculum of each individual species was prepared in MRS (or in this same medium containing sodium chloride as a sodium source) and the cells were cultivated, overnight, at 37ºC with an orbital agitation of 100 rpm. On the next day, these cells were used to inoculate (at an OD_{600nm} of 0.4 for *L. gasseri* and 0.1 for the two *Candida* species) fresh MRS medium. Growth in this co-culture system was accompanied for 4 days, at 37ºC and using an orbital agitation of 100 rpm, by following the increase in cellular viability of the two species based on the number of colony-forming units (CFUs). For this, aliquots of co-cultures were taken, serially diluted, and plated on solid MRS supplemented with 96 mg/L fluconazole (an antifungal concentration that fully prevented growth of *Candida* colonies and thus only *L. gasseri* colonies were visible) or in YPD supplemented with 300 mg/L tetracycline (an antibiotic concentration that fully prevented the growth of *L. gasseri* colonies and therefore only *Candida* colonies were visible). The number of *Candida* colonies formed onto the surface of YPD plates was counted after 2 days of incubation at 30ºC, while the number of *L. gasseri* colonies formed onto the surface of MRS plates was counted after 2 days of plate incubation at 37ºC in a Genbox (Biomerieux) with a

candle inside to assure microaerophilia[541, 542]. As controls, it was performed single-cultivations of *L. gasseri*, *C. albicans* and *C. glabrata* in the same conditions used for the co-cultivations. Quantification of the amounts of lactic acid, acetic acid or glucose present in the broth during single or multi-species cultivation was performed by HPLC (equipped with an UV detector, for quantification of lactic and acetic acids, and with a RI detector, for quantification of glucose) using an Aminex HPX87H (Biorad®) column and 0.005M H_2SO_4 (at a flow rate of 0.6 mL/min of) as eluent.

II.3.3. Co-cultivation of *L. gasseri* with *C. glabrata* or *C. albicans* under biofilm-forming conditions

To examine growth under biofilm-forming conditions of *L. gasseri*, alone or in co-cultivation with *C. albicans* or *C. glabrata*, a pre-inoculum of each species was prepared in MRS (or in this same medium containing sodium chloride as a sodium source) and the cells were cultivated, overnight, at 37ºC, using an orbital agitation of 100 rpm. These pre-cultures were used to inoculate 200 µL of fresh MRS in plastic µ-slide 8 well plates (Ibidi) so that the initial cell densities (estimated based on OD_{600nm}) were 10^6 CFU/mL for the two *Candida* species and 10^8 CFU/mL for *L. gasseri*. After 24h of cultivation at 37ºC with 25 rpm agitation, the supernatant of the single or co-cultures was removed and the biofilm formed was washed with 200 µL of PBS. To assess cellular viability in the single- or multi-species biofilms formed, 3 µM of SYTO 9 Green Fluorescent Nucleic Acid Stain (Molecular Probes, Eugene, OR, USA) was added to the single or co-cultures and the cells were left in the dark for 30 minutes. After this time, 4 µM TO-PRO-3 iodide (Molecular Probes, Eugene, OR, USA) was added and the cultures were incubated under the same conditions for another 15 minutes. The gain adjustment in each channel was optimized (and kept during the experiments) taking into account the intensity fluorescence signal of live and dead single cells. Live single cells were stained directly after growth, while dead single cells were prepared by heating a cell sample at 65ºC for 10 minutes in a dry bath. Then, single or multiple species biofilms were imaged by confocal laser scanning microscopy using a Leica TCS SP5 inverted microscope with a 63x water (1.2 numerical aperture) apochromatic objective. Cells were imaged with the 488 nm Ar^+ laser line to detect cells stained with SYTO 9 (emission collected at 500 – 590 nm) and with the 633 nm He-Ne laser line to detect cells stained with TO-PRO-3-Iodide (emission collected at 645-795 nm), a setup that minimizes cross-interference between the two channels as described in Pinto *et al.*, 2019[543]. Scanning electron microscopy (SEM) was performed similarly to the aforementioned protocol but biofilms were prepared in 2.5 mL MRS in 5 mL polystyrene plates. After 24h of incubation at 37ºC with 25 rpm agitation, the supernatant was removed and the cells adhered to the surface of the plate were dehydrated using the following protocol: washing with distilled water; washing with 70% ethanol for 10 minutes; washing with 95% ethanol for 10 minutes; and washing with 100% ethanol for 20 minutes. Plates were then dried in a desiccator for at least 96h and visualized by scanning electron microscope (SEM) using a magnification between 2000x and 35000x with a high voltage of 20.0 kV.

II.3.4. *Candida* spp. adhesion to vaginal epithelial cells

Adhesion of *Candida* spp. and *L. gasseri* to the vaginal epithelial cells was performed by single and co-cultivating these species together with VK2/E6E7 human epithelial cells. These epithelial cells were cultivated and inoculated in 24-well polystyrene plates (Greiner) at a density of 1.25×10^5 CFU/well. To initiate the assays, the culture medium of mammalian cells was removed and replaced by new culture medium in each well. Subsequently, the wells were also inoculated with *C. albicans* or *C. glabrata* and *L. gasseri* at a density of 1.25×10^6 CFU/well, corresponding to a multiplicity of infection (MOI) of 10. Afterwards the plate was incubated at 37°C under 5% CO_2 for 3 hours. After this time, the culture supernatant (corresponding to the non-adhered cell fraction) was collected and each well (containing the cells) was washed 3 times with 500 µL of phosphate-buffered saline (PBS) at pH 7.4, after which the cells were further incubated, for 15 minutes at room temperature, with 500 µL of 0.5% Triton X-100. To determine the number of adhered and non-adhered *Candida* or *L. gasseri* cells appropriate aliquots of the non-adhered and adhered cell fractions were plated in solid MRS supplemented with 96 mg/L fluconazole (to estimate *L. gasseri* abundance) or in YPD supplemented with 300 mg/L tetracycline (to estimate *Candida* spp. abundance). The number of *Candida* colonies formed onto the surface of YPD plates was counted after 2 days of incubation at 30ºC, while the number of *L. gasseri* colonies formed onto the surface of MRS plates was counted after 2 days of plate incubation at 37ºC in a Genbox (Biomerieux) with a candle inside to assure microaerophilia[541, 542]. As controls, we performed incubations of the mammalian cells with *L. gasseri* and *C. glabrata* or *C. albicans* alone, in the same conditions used for the co-cultivations.

II.4. Results

II.4.1. Co-cultivation of *C. glabrata* and *C. albicans* with *L. gasseri* results in decreased viability of the yeasts, either under planktonic or biofilm-forming conditions

The majority of the studies that examined the interference established between the vaginal species *L. gasseri* and *Candida* focused on the inhibitory effect prompted by supernatants obtained from bacterial cultures[218, 388, 389], usually obtained in MRS, the canonical growth medium used for lactobacilli[544, 545]. Considering that *in vivo* these two species are in close contact, this work focused on their direct co-cultivation in planktonic or in biofilm-forming conditions. To examine planktonic growth, the two *Candida* species (*C. albicans* and *C. glabrata*) and *L. gasseri* ATCC 33323 were cultivated in liquid MRS for 96h (Figure II.1). To simulate the higher abundance of lactobacilli in the vaginal microflora, compared with the one of *Candida*[49, 546-548], these co-cultivations were started using ~100 more bacterial than yeast cells (~10^8 CFUs/mL *L. gasseri* compared to 10^6 CFUs/mL of *C. albicans* or *C. glabrata* - Figure II.1). Under the experimental conditions used for the co-cultivation (100 rpm of agitation and 37ºC) the yeast cells resumed growth immediately after re-inoculation and maintained it until 24h, after which they entered stationary phase (Figure II.1). The bacterial population also increased, although much less, likely due to a higher number of inoculated cells, compared with the one use for *Candida* (Figure II.1). Consistent with co-cultivation being a more competitive and challenging environment, the growth rate of *C. glabrata* and *C. albicans* in the presence of *L. gasseri* decreased by 32% and 33% respectively, compared to the values observed in single-culture (0.25h^{-1} obtained in single-culture of *C. glabrata*, compared to 0.17h^{-1} in co-culture; 0.24h^{-1} obtained in single-culture of *C. albicans*, compared to 0.16h^{-1} obtained in co-culture). Besides a reduction in the growth rate, it was also noticeable that co-cultivation with *L. gasseri* induced a prominent decrease (ranging between 53 and 98%) in the cellular viability of *C. albicans* and *C. glabrata*, this being considerably more prominent for the first species (Figure II.1). No significant reduction in viability was observed in the two single-cultures of *Candida* indicating that the loss of viability observed in the co-culture setting is a direct effect of the presence of *L. gasseri* (Figure II.1). Differently, co-cultivation increased the cellular viability of *L. gasseri* up 10^7 CFUs/mL, compared to 10^5 CFUs/mL attained in single-culture (Figure II.1). In agreement with our results, reduced viability of *L. gasseri* cells upon short-medium term cultivation in MRS has been reported in other studies, presumably due to autolysis[549-554]. In order to exclude strain-dependent effects, we have repeated the co-cultivations using four vaginal *C. glabrata* and *C. albicans* strains and in both cases, it was clear that co-cultivation with *L. gasseri* resulted in reduced viability of the yeast strains (see results in Annex Figure II.1). However, it was interesting to observe that the decrease in viability of the vaginal *Candida* strains imposed by co-cultivation with *L. gasseri* was smaller than the one obtained with the reference strains (compare results in Annex Figure II.1 and Figure II.1).

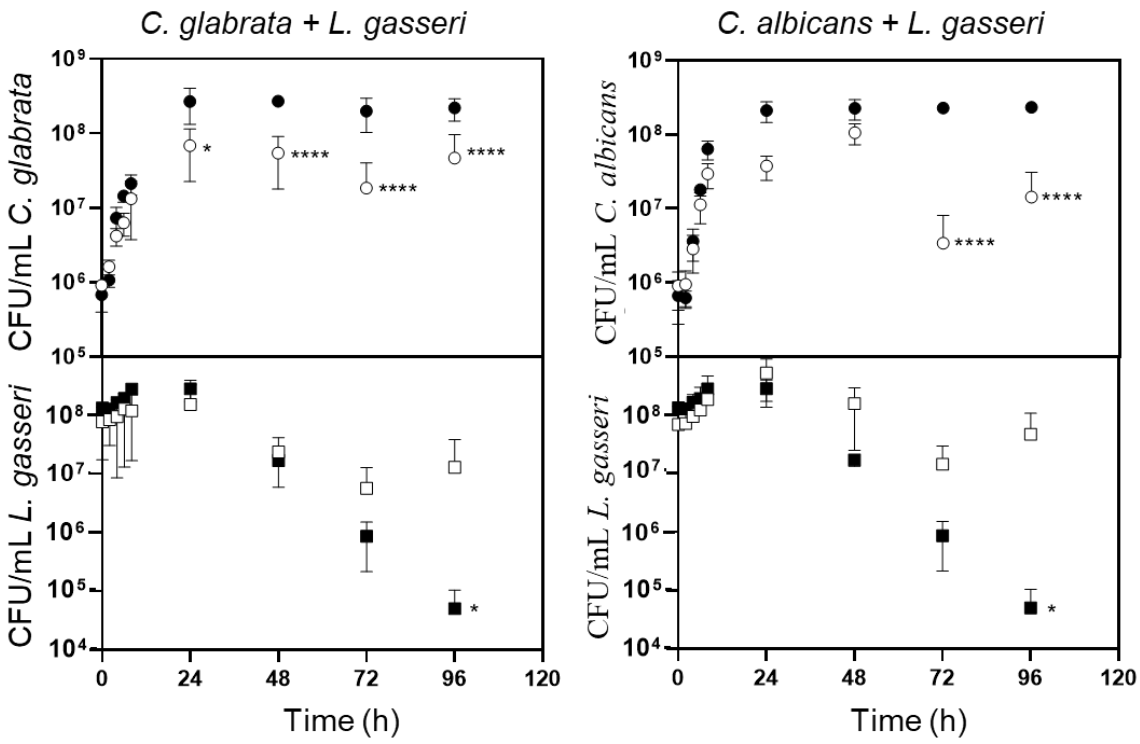


Figure II.1. Cellular viability of *C. glabrata*, *C. albicans* and *L. gasseri* along single or co-cultivation in MRS medium. After inoculation, cells of *C. albicans* (○,●), *C. glabrata* (○,●) or *L. gasseri* (□,■) were cultivated at 37ºC and 100 rpm for 96h with the growth of the different species being accompanied based on cellular viability, as detailed in materials and methods. Filled symbols correspond to the samples taken during single-species cultivation while open symbols corresponds to the samples taken during co-cultivation. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

To test the effect of co-cultivation under sessile conditions the same experimental setting was used with the difference that *C. albicans*, *C. glabrata* and *L. gasseri* were cultivated in 8-well microplates for confocal microscopy or small Petri dishes suited for scanning microscopy (SEM) analysis. After 24h of cultivation, it was possible to observe that *C. albicans* and *C. glabrata* formed a mixed biofilm with *L. gasseri* involving very close cell-to-cell contacts, as shown by the scanning electron microscopy (SEM) images depicted in Figure II.2.

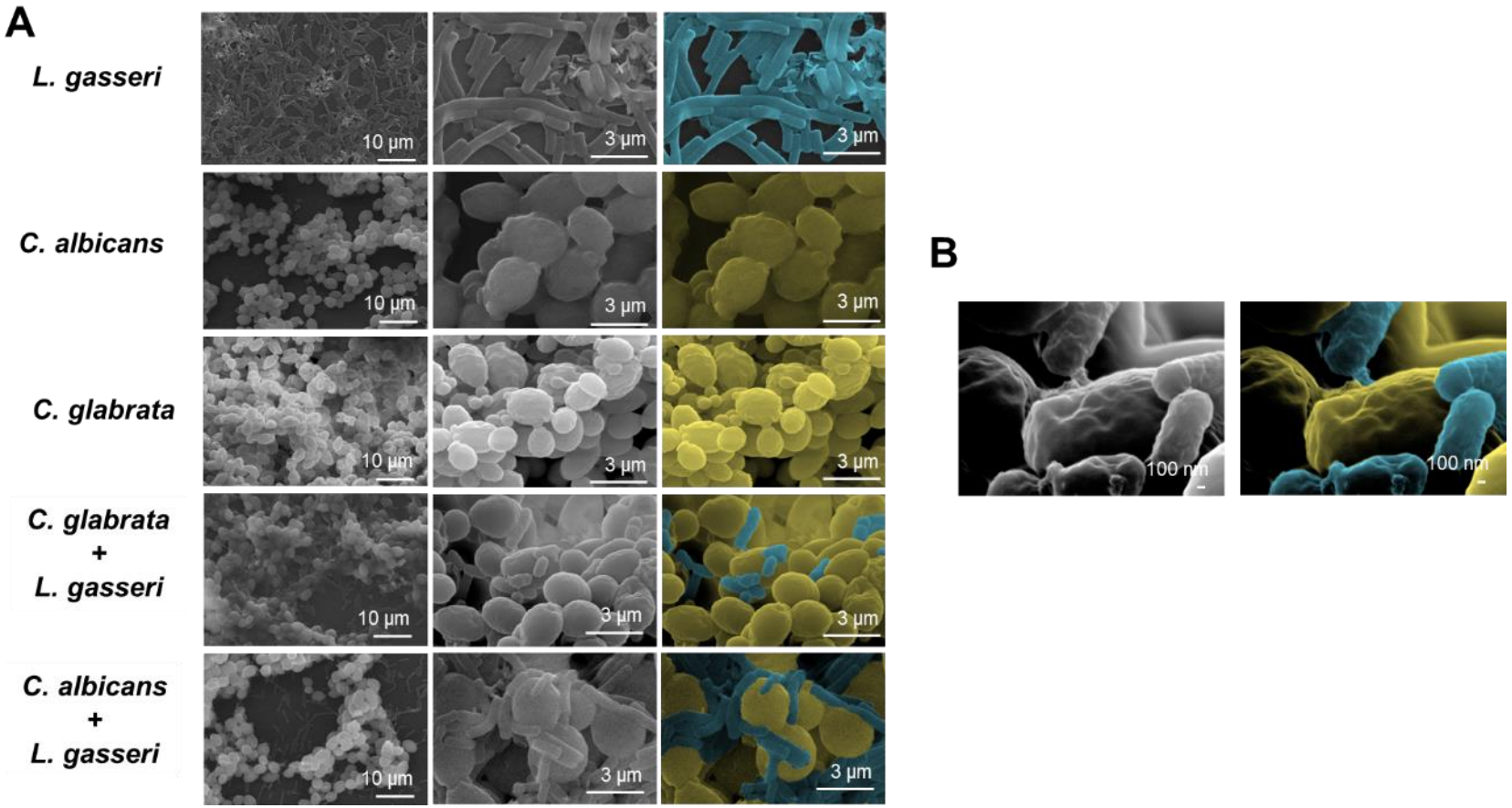


Figure II.2. (A) Biofilms formed by *L. gasseri*, *C. albicans* and *C. glabrata* during single or co-cultivation in MRS, as detected by scanning electron microscopy. The biofilms were obtained using the same conditions described in materials and methods, with the exception that was used 5 mL-polystyrene plates instead of the 8 mm plates used in the fluorescence microscopy imaging. These images were obtained after 24h of cultivation at 37ºC and 25 rpm using a magnification of 2000x to 35000x over the dried biofilms. For the sake of facilitating identification of cells, in the last column, the yeast cells were labelled in yellow, while the bacterial cells were labelled in blue. **(B)** Image obtained at the nanoscale clearly shows the close cell-cell contacts established between *L. gasseri* and *C. glabrata*. The image is representative of others also found while examining the imaged field.

Single-species biofilms formed by *C. albicans* exhibited what appeared to be a multi-layered structure, while those formed by *C. glabrata* appeared more disperse (Figure II.3). These differences in the structural organization of the biofilms formed by these two yeasts are consistent with results reported in other studies[320, 555-557]. No significant differences were obtained in what concerns height of the single-species and multi-species biofilms, with the exception of the biofilms formed by *L. gasseri* alone that were considerably thinner (Annex Figure II.2). To understand what could be the outcome of the formation of these mixed biofilms in terms of viability, we took advantage of SYTO9 and TO-PRO3 iodide labelling that allowed us to differentiate, the yeast from the bacterial cells, while also distinguishing viable from non-viable cells directly in the biofilm[558]. In Figure II.3 is shown the results of this labelling in single- and in multi-species biofilms. The results clearly demonstrate that the proportion of non-viable cells (labelled in red) was much higher in the mixed-biofilms than in the single-species ones, this effect being clearly more evident in the mixed biofilms formed between *L. gasseri* and *C. glabrata* (Figure II.3). Closer inspection of the images shows that these non-viable red-labelled cells correspond almost entirely to the yeasts while no significant loss of viability was observed for the *L. gasseri* cells (Figure II.3). To get a more quantitative view, was imaged in close detail a set of pictures (corresponding to more than 1000 yeast cells per condition) and found that the number of non-viable *C. glabrata* cells is about 2.6-fold higher in the mixed-biofilms than in the biofilms formed in the absence of the bacterium (Figure II.3). In the case of *C. albicans* the number of non-viable cells in the mixed-biofilm increased roughly 2-fold (Figure II.3). A striking labelling of TO-PRO-3 iodide was observed in the single-species *L. gasseri* biofilms visualizing at higher magnification that the labelling corresponds to what appears to be the extracellular matrix (Figure II.3 and a magnification shown in Annex Figure II.3). Upon entry into microbial cells, TO-PRO-3 iodide is described to bind nucleic acids[558, 559] and thus it is possible that the observed labelling results from accumulated extracellular DNA (eDNA), as this was described to occur in biofilms formed by other lactobacilli species[560-562]. Notably, such labelling pattern was not detected in the single-species biofilms formed by the two *Candida* species, albeit eDNA has been reported in the extracellular matrix of *C. albicans* biofilms[563].

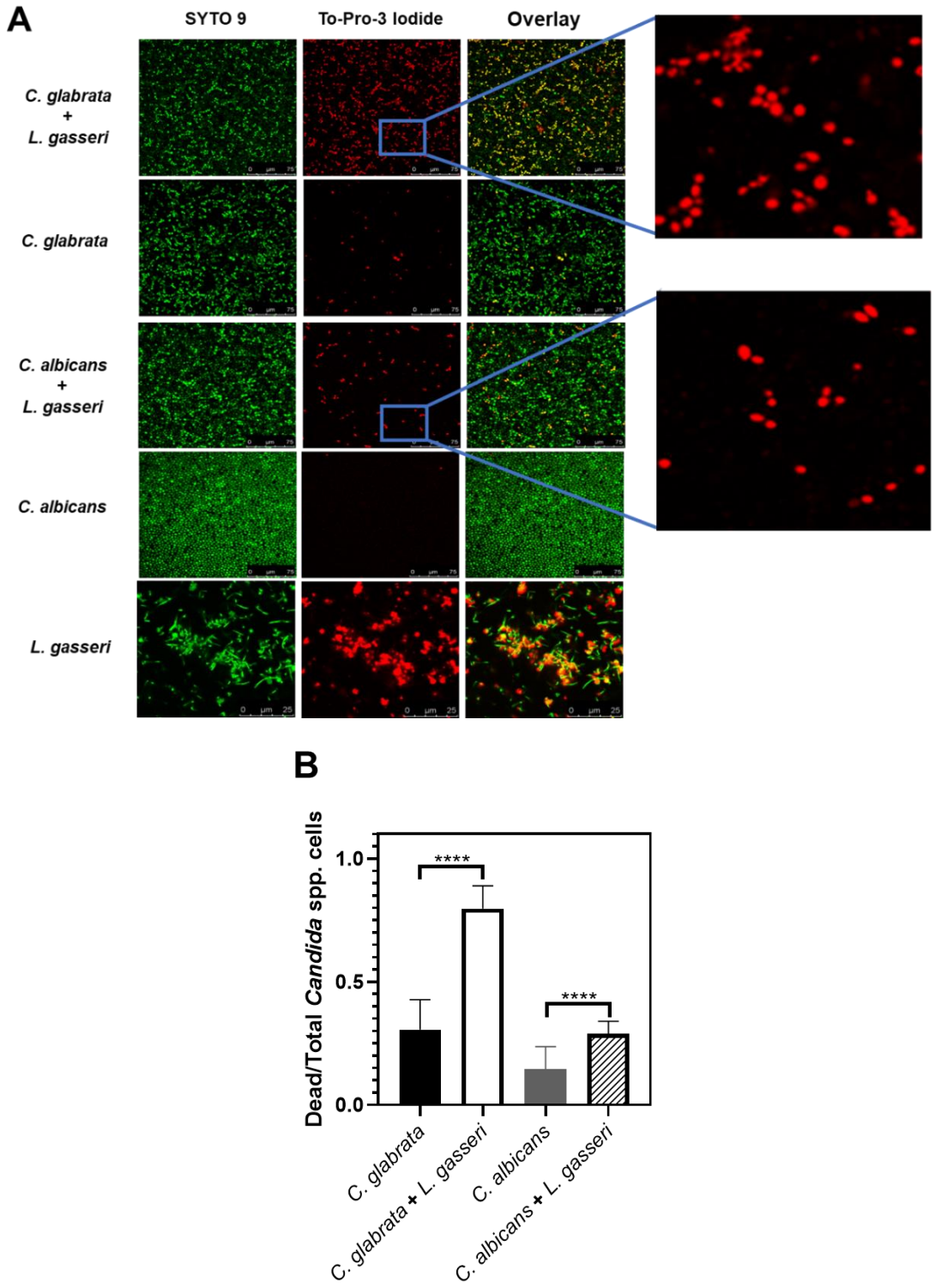


Figure II.3. (A) Live/dead imaging of cells in single-species or in mixed biofilms formed by *L. gasseri*, *C. albicans* or *C. glabrata* after 24h of cultivation, at 37ºC and 25 rpm, in MRS. The insert details the labelling of the dead yeast cells while in the mixed biofilms. The images presented are representative of a set taken from the biofilms in three replica experiments performed; *Candida* spp. single and mixed biofilms scale bar corresponds to 75 µm while *L. gasseri* single biofilm scale bar corresponds to 25 µm; **(B)** Quantification of the number of dead *Candida* cells in the single-species or in the multi-species biofilms formed, based on quantification of the number of red-labelled yeast cells in all pictures taken from the biofilms, compared to the total number of *Candida* cells in the field (corresponding to green-labelled cells). For this quantitative analysis, more than 1000 yeast cells were imaged in each condition. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

We have also tested the ability of *L. gasseri* to reduce the adhesion of *Candida* spp. on the surface of vaginal epithelial cells, this being the first step in the process of a successful biofilm formation. The results show in Figure II.4 revealed that the adhesion of the yeasts cells to vaginal epithelial cells is significantly reduced when the yeasts are cultivated in the presence of *L. gasseri*, while adherence of bacterial cells is not different in the single or in the co-culture setting. Specifically, we observed that in co-cultivation with *L. gasseri*, the number of *C. glabrata* or *C. albicans* cells adhered to the surface of vaginal cells was reduced 59% and 66%, respectively (Figure II.4). This result suggest that *L. gasseri* not only severely impacts formation of *C. glabrata* and *C. albicans* biofilms in abiotic but also in biotic surfaces.

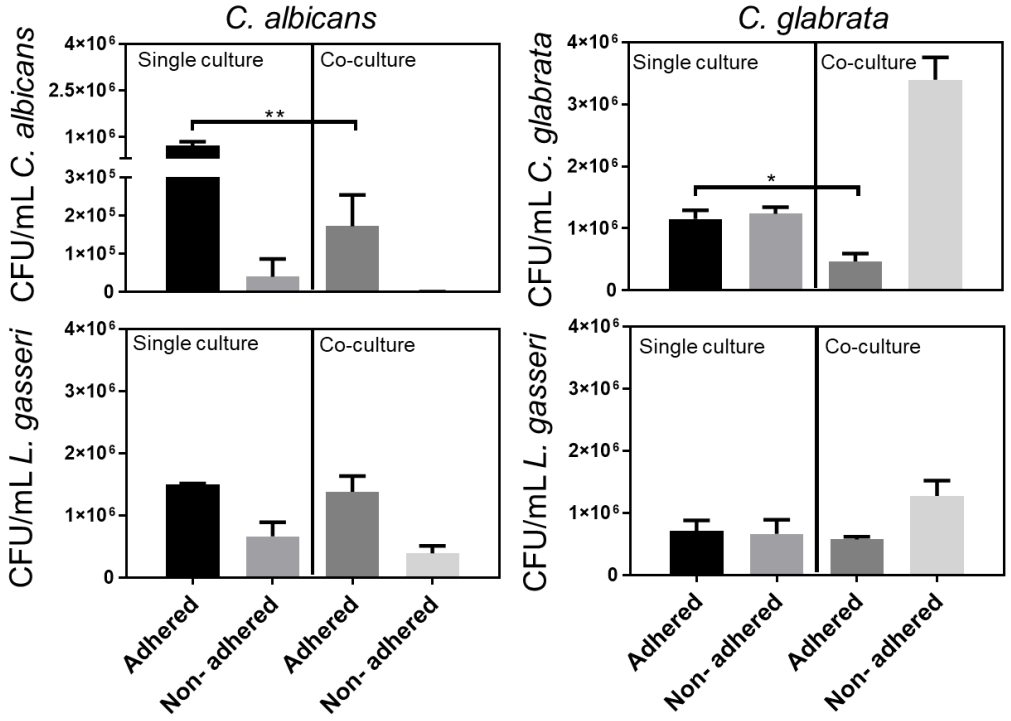


Figure II.4. *L. gasseri* reduces the adhesion of *C. glabrata* and *C. albicans* to vaginal epithelial cells. Columns represent the colony forming units (CFUs) of *Candida* spp. and *L. gasseri* adhered and non-adhered to vaginal epithelial cells in single and co-cultivation. Cultivation was performed at 37°C under 5% CO_2 for 3 hours. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

II.4.2. While in co-cultivation with *L. gasseri*, *C. albicans* and *C. glabrata* buffer the acidification prompted by the bacterium with consequences in the equilibrium of ionizable species like lactic and acetic acids

Taking into account that co-cultivation increased the viability of *L. gasseri*, while it decreased the viability of the two *Candida* species (Figure II.1), and that the high autolysis of *L. gasseri* in MRS could be associated with the low pH of the medium (presumably due to the lactic acid formed)[552], it was decided to monitor the pH achieved in single and in co-cultures. HPLC analyses of the broth confirmed the expected production of lactic acid by *L. gasseri* in amounts

that ranged between 10 g/L in the single-culture to approximately 5 g/L in the co-culture (Annex Figure II.4 panel A). The lower amount of lactic acid produced in the co-cultures was consistent with a more rapid depletion of glucose in the fermentation medium, likely resulting from the yeast's metabolic activity (Annex Figure II.4 panel A). As expected, no production of lactic acid was observed in the supernatants obtained from single-cultures of the two *Candida* species, only an accumulation of ethanol was detectable (results not shown), which is compatible with the microaerophilic setting used. Concomitant with the production of lactic acid, a continuous acidification of the fermentation broth was observed, both in the single and in the co-cultivation settings (Figure II.5 panel A). While in the single-cultures of *L. gasseri*, the acidification persisted along the entire 96h time frame and reaching a final pH of about 4, in the co-culture the pH started to increase after an initial drop (Figure II.5 panel A). These observations suggest that the metabolic activity of the two *Candida* species buffers the acidification prompted by the accumulation of lactic acid, this buffering capacity being higher for *C. glabrata* than for *C. albicans* (final pH of the co-cultures was around 6.8 and 5, respectively; Figure II.5 panel A).

One of the factors imparted by the differences in pH of the broth registered during the single or co-cultivation of *C. albicans*/*C. glabrata* with *L. gasseri* is the distribution between the dissociated ($RCOO^-$) and undissociated form (RCOOH) of lactic acid, as well as of other ionizable species accumulated in the broth, this necessarily depending on the corresponding pKa values. Using the Handerson-Hasselbach equation to estimate the ratios between the amounts of lactate and lactic acid achieved during single or co-cultivation with *Candida*, it is clear that most of the lactic acid accumulated in the broth of the co-culture was dissociated (Figure II.5 panel B). In the single-cultures of *L. gasseri* lactate also prevails, however, the amount of undissociated lactic acid is considerably higher than the one present in the broth of co-cultures (Figure II.5 panel B). Besides lactic acid, the ratio of dissociated and undissociated acetic acid was also computed since the MRS medium contains ~60 mM of sodium acetate supplied as a sodium source (Figure II.5 panel B). In this case, there was a marked difference between the result obtained in single and co-cultures of *L. gasseri*, as undissociated acetic acid clearly predominated in the single-culture, while in the co-culture acetate prevailed (Figure II.5 panel B). These observations show that the co-cultivation of *C. glabrata* or *C. albicans* with *L. gasseri* modulates important aspects of the composition of the supernatant including its pH and, consequently, the acid-base equilibrium of ionizable species like lactic and acetic acids.

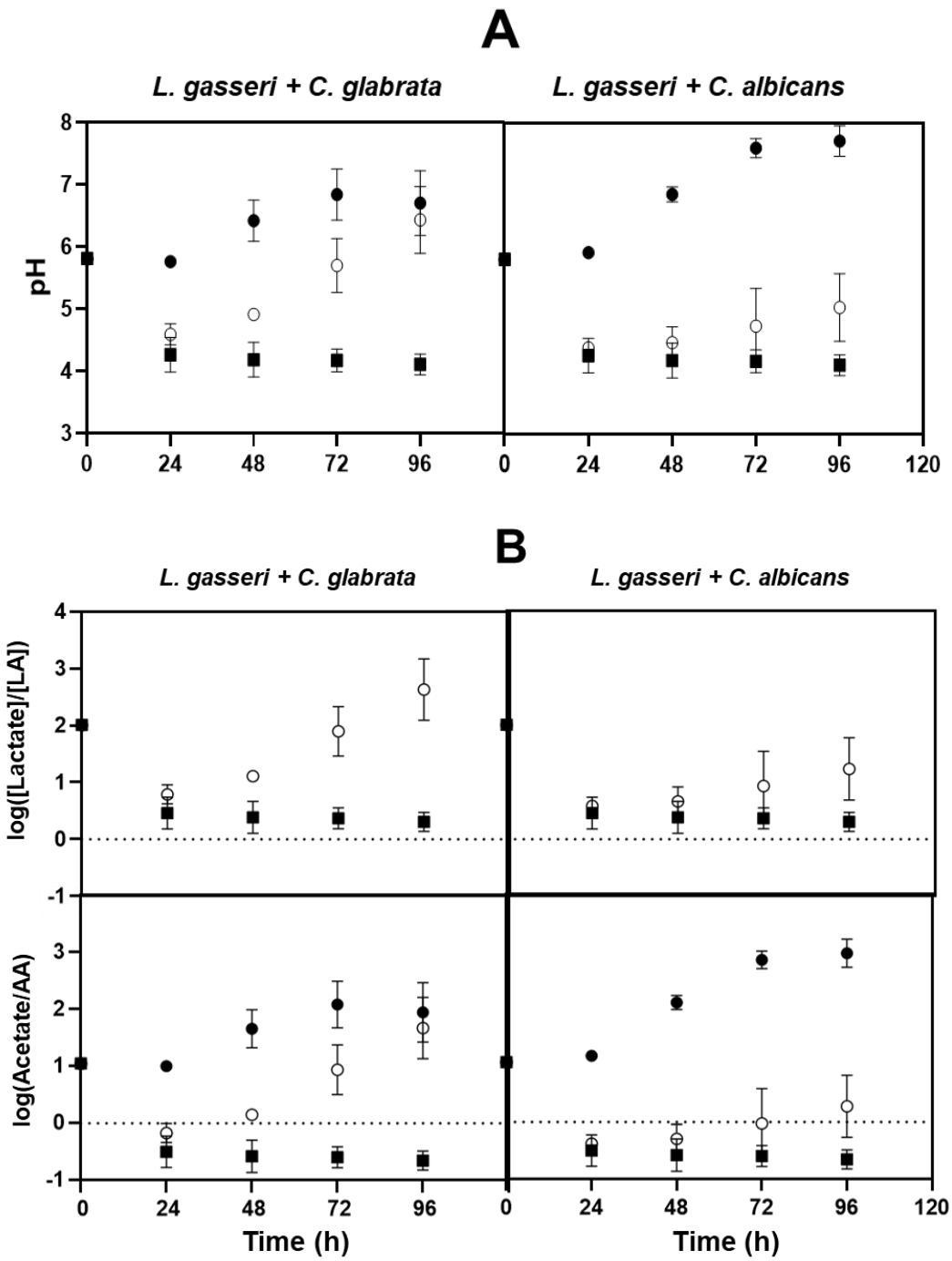


Figure II.5. (A) Variation of medium pH during cultivation of *L. gasseri* (□,■) in MRS, alone or in combination with *C. glabrata* or *C. albicans* (○,●), under the same conditions as those used to obtain the growth curves shown in Figure II.1. In panel **(B)** the variations in the ratio of lactate/ lactic acid (LA) and acetate/acetic acid (AA) are shown along the single-species or co-cultivation settings, as estimated by the Handerson-Hasselbach equation and using the pHs determined in panel A, a pKa of 3.86 for lactic acid and a pKa of 4.76 for acetic acid. Filled symbols correspond to the samples taken during single-species cultivation while open symbols correspond to the samples taken during co-cultivation. The results presented are representative of, at least, three independent replicas.

II.4.3. Acetate augments anti-*Candida* activity prompted by *L. gasseri* cells

Our results shown in Figure II.5 panel B demonstrate that in co-cultures of *L. gasseri* with *C. albicans* or *C. glabrata* the sodium acetate supplied in the MRS medium prevails as in the acetate form. Considering that acetate has been demonstrated to induce the expression of bacteriocins in several Gram-positive species[564, 565], was hypothesized whether it could also modulate the anti-*Candida* effect exhibited by *L. gasseri*. For this was co-cultivated *L. gasseri* with *C. glabrata* in MRS medium under the same conditions used before but replacing sodium acetate by the same amount of sodium chloride. This replacement resulted in an accelerated loss of viability of *L. gasseri* cells while in single-culture, a phenotype that was rescued when the bacterial cells were cultivated in the presence of the yeasts (Figure II.6 panel A). Remarkably, despite the maintenance in the viability of *L. gasseri* population, no significant loss of viability of *C. glabrata* could be detected in the co-cultures performed in MRS with NaCl (Figure II.6 panel A). Notably, the capability of acetate to enhance the ability of *L. gasseri* cells to reduce the viability of *C. glabrata* cells was concentration dependent and still detectable at concentrations as low as 4 mM (Figure II.6 panel B and Annex Figure II.5). Consistent with the results observed for *C. glabrata*, co-cultivation of *L. gasseri* with *C. albicans* cells in MRS medium having NaCl (and not sodium acetate) as the sodium source also resulted in the incapability of the bacterium to induce loss of viability in the yeast cell population (Annex Figure II.6). The replacement of sodium acetate by increasing amounts of sodium chloride led to a reduction in the production of lactic acid (up to a maximum of 50%) prompted by the bacterium (Annex Figure II.4 panels B and C). While in single-cultures, this decrease can be attributed to the lower viability of the bacterial cells when growing in MRS-NaCl, compared to MRS (Figure II.6 panel A and Figure II.1), in the co-cultures performed in MRS-NaCl or in MRS the viability of *L. gasseri* cells was identical and therefore the lower production of lactic acid is likely to result from the lower availability of carbon (Figure II.6 panel A and Figure II.1). The capability of acetate to increase the anti-*Candida* potential of *L. gasseri* was also detected when a vaginal clinical strain of the bacterium was used (*L. gasseri* ISTLg97) confirming that the effect is not exclusive for the used reference strain (Annex Figure II.7).

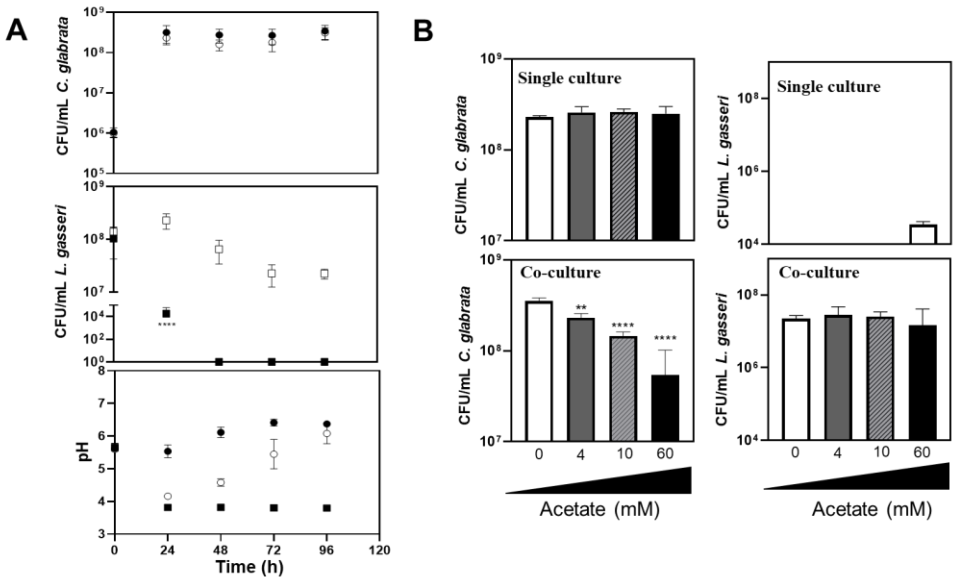


Figure II.6. The acetate present in MRS medium potentiates the inhibitory effect of *L. gasseri* over *C. glabrata* cells. **(A)** Cellular viability and medium pH during single or co-cultivation of *C. glabrata* (○,●) with *L. gasseri* (□,■) in MRS medium having 60 mM sodium chloride as the sodium source (instead of the normally used sodium acetate). The cells were cultivated, alone or in the presence of each other, in the same conditions described in Figure II.1. Filled symbols correspond to the samples taken during single-species cultivation while open symbols correspond to the samples taken during co-cultivation.; **(B)** Cellular viability of *C. glabrata* and *L. gasseri* after 96h of cultivation, in single-culture or in co-culture, in MRS media having increasing concentrations of acetate. Note the decreasing viability of the yeast cells as the concentration of acetate increases. The results shown in this panel concerning the cellular viability of the different microbial species were taken from the full growth curves that are shown in Annex Figure II.5. Statistical significance of the differences found in the presence or absence of acetate was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

II.4.4. The effect of acetate in augmenting anti-*Candida* activity in *L. gasseri* cells is also observed in biofilm-forming conditions

The above reported effect of acetate in enhancing the capability of *L. gasseri* cells to induce loss of viability in *C. albicans* and in *C. glabrata* prompted us to examine whether the same effect could also be detected in biofilms. For that, was used the same experimental setting as used above to detect the formation of mixed biofilms between *L. gasseri* and *C. albicans* or *C. glabrata*, including the live-dead confocal microscopy imaging. The modulation of acetate concentration in the MRS medium did not significantly alter the height of the biofilms formed (Annex Figure II.2). On the other hand, a marked increase in the viability of *C. glabrata* and *C. albicans* in mixed biofilms formed with *L. gasseri* during cultivation in MRS without acetate was observed, markedly contrasting with the high loss of viability that was observed in the normal composition of this medium including 60 mM acetate (Figure II.7 and Annex Figure II.8). Also as observed under planktonic conditions, this effect of acetate in augmenting the anti-*Candida* activity of *L. gasseri* cells was concentration-dependent (Figure II.6). Strikingly, the unusual TO-PRO-3-labelling registered in the biofilms formed by *L. gasseri* cells was no longer detected when acetate was removed from the medium (Figure II.7).

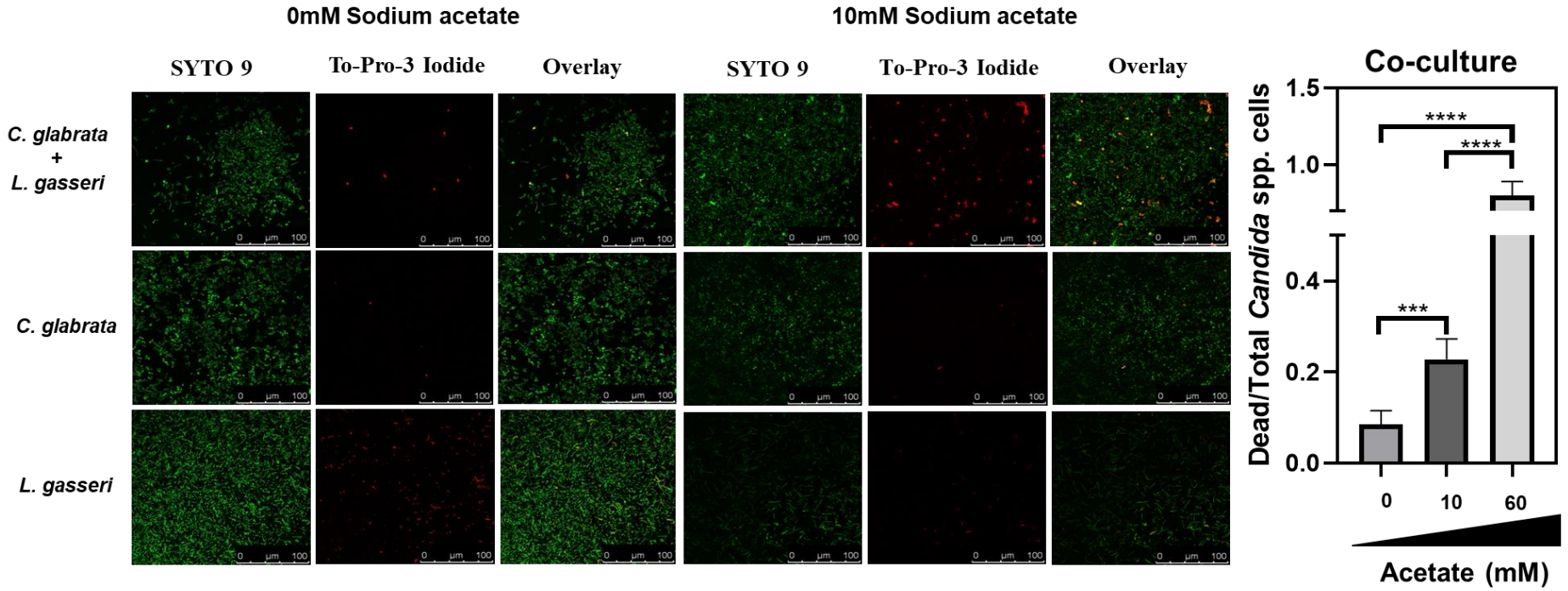


Figure II.7. Effect of acetate on the capability of *L. gasseri* to induce loss of *C. glabrata* cellular viability in mixed biofilms. Biofilms formed by *L. gasseri* and *C. glabrata* after 24h of single or co-cultivation in MRS medium with increasing amounts of acetate at 37ºC and 25 rpm were imaged by fluorescence confocal microscopy to distinguish between live and dead cells, as detailed in materials and methods. The pictures presented are representative of a cohort obtained in three independent replicas and that were used to quantify the number of dead *C. glabrata* cells (labelled in red) compared to the total number of *C. glabrata* cells in the field (labelled in green and distinguishable from the bacterial cells based on their yeast-like morphology). All scale bars corresponds to 100 µm. The result of the quantification is shown in the chart presented at the right. The data presented concerning the number of *C. glabrata* cells in mixed biofilms formed in MRS having 60 mM acetate is the one shown in Figure II.3. Statistical significance of the differences obtained in the number of dead yeast cells in the different conditions was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

II.5. Discussion

This chapter focused on the interaction established between the poorly studied vaginal species *L. gasseri* and the pathogenic yeasts *C. albicans* and *C. glabrata*, both being frequent colonizers of the female vaginal tract. Other studies described the inhibitory potential of lactobacilli species, including of *L. gasseri*, against *Candida* and other vaginal pathogens[218, 387, 390]. However, these studies don't explore co-cultivation describing instead relevant alterations in pathophysiological traits of *Candida* when cultivated in media supplemented with various amounts of supernatants obtained from bacterial cultures[218, 388, 389, 566]. The results herein shown demonstrate that the direct co-cultivation of *L. gasseri* with *C. albicans* and *C. glabrata* has outcomes that cannot be fully recapitulated by the bacterial supernatants themselves. For example, co-cultivation of *C. albicans*/*C. glabrata* with *L. gasseri* drastically altered the pH of the broth with consequences in the acid-base equilibrium of ionizable species like the organic acids acetic and lactic acid. In particular, we found that during co-cultivation, the two yeasts counteracted the prominent acidification of the medium promoted by the accumulation of lactic acid produced by *L. gasseri* cells. Consequently, the final pH of the co-cultures was ~6 after 96h, while in the single-cultures of *L. gasseri* this pH was about ~4.1. Prior studies have shown the capability of *C. albicans* to alkalinize the medium (via ammonium excretion) when using amino acids[567, 568] or carboxylic acids as carbon sources[567, 568]. *C. glabrata* has also been shown to alkalinize the medium while using amino acids as carbon sources but the identity of the buffering compound was not disclosed[294]. Notably, in these studies, the alkalinization of the medium prompted by *C. albicans* or *C. glabrata* occurred under glucose limiting conditions, which is in line with the results we had obtained since the pH started to increase after 48h, when no glucose was available in the broth (Annex Figure II.4). This capability of *C. albicans* and *C. glabrata* to induce alkalinization under acidic conditions has been linked with their increased ability to thrive in the highly acidic phagolysosome, favouring colonization and immune evasion[294, 569]. In *C. albicans* the alkalinization has also been shown to auto-induce hyphal morphogenesis[568]. It is possible that *in vivo*, when present in the vaginal tract, *Candida* cells can also counteract the acidification prompted by lactobacilli. Among other outcomes (such as the promotion of yeast-hyphae transition) this buffering also avoids the accumulation of undissociated organic acids in the environment that have a potent antimicrobial effect, including against *Candida*[430, 570, 571]). Besides lactic acid, acetic acid, 4-hydroxyphenylacetic, succinic, butyric and formic acids are other organic acids present in the vaginal fluid[130, 411, 572] and whose chemical dissociation can be impacted by changes in pH.

The modulation of pH observed to occur along co-cultivation of *Candida* with *L. gasseri* shows that the use of supernatants from bacterial cells may not be a good proxy to study the interaction since the amount of undissociated acids is much higher than the one obtained in a co-culture supernatant creating confounding effects (is the inhibition caused by the lactobacilli or by the accumulation of undissociated organic acids?). The use of bacterial supernatants obtained from cultivation in MRS is particularly problematic since these will invariably contain toxic amounts

of acetic acid. In this context, some of the inhibitory effects reported in growth and virulence traits of *Candida* upon exposure to lactobacilli supernatant cultures result, in fact, from the effects of acetic acid. In line with our results, the acetate present in MRS medium was described to have antifungal properties when used in synergy with *L. rhamnosus*[573]. Collectively these findings increase the relevance of using experimental settings based on contact of the species as they appear to establish dynamic interactions that may go beyond the mere accumulation of metabolites in the broth and, therefore, are not fully phenocopied by bacterial supernatants.

Co-cultivation of *C. albicans* or *C. glabrata* with *L. gasseri* resulted in decreased viability of the two yeasts (and this is induced by the bacterium and not by accumulated organic acids since we demonstrated that in co-cultures the non-toxic acetate and lactate forms prevailed), either in planktonic or in biofilm-forming conditions. Previous studies showed that biofilm formation by *C. albicans* is impacted by co-cultivation with the vaginal species *L. crispatus*, *L. jensenii* and *L. iners*[140, 387], however, as the biofilms formed were not microscopically observed the authors did not conclude about the capacity of these species to interact with one another. Both *C. albicans* and *C. glabrata* had been described to form mixed biofilms with other bacteria including *Streptococcus mutans*[574], *Staphylococcus aureus*[575] or *Pseudomonas aeruginosa*[576], but to the best of our knowledge, this is the first report involving *L. gasseri*. In the biofilms, the *C. glabrata* cells were highly susceptible to the presence of *L. gasseri*, while the reduction of viability of *C. albicans* cells was considerably lower (opposite to what was observed under planktonic conditions). We cannot rule out that the mechanism of inhibition prompted by *L. gasseri* in a biofilm may differ from those imparted in planktonic growth as close cell-cell contacts (well demonstrated to occur in Figure II.2) may trigger specific responses. In this context, it is interesting the previous demonstration that *L. paracasei* cells respond to direct contact with *S. cerevisiae* cells[577]. Another hypothesis is that the access of *L. gasseri* to *C. glabrata* cells in the mixed biofilm can be higher due to a stiffer structure of the *C. albicans* biofilms caused, among other aspects, by high amounts of extracellular matrix[578, 579]. The higher sensitivity of *C. glabrata* to *L. gasseri* in the mixed biofilm is interesting since this species is much less frequently isolated from the vaginal tract than *C. albicans*[256, 580, 581], although it is known to be very resilient to environmental stress[257, 330, 430].

The fact that the anti-*Candida* activity prompted by *L. gasseri* cells depends on the presence of acetate in a concentration-dependent manner is a novel finding of our work. *In vivo,* acetate is present in the vaginal fluid due to the metabolic activity of colonizing microbes[21, 411, 582] and thus it is possible that it contributes to maintain the interference of *L. gasseri* over *Candida*. Note that the potential of acetate in augmenting the virulence of *L. gasseri* towards *Candida* cells was detectable at 4 mM acetate, a concentration within the range found in vaginal fluid of a lactobacilli-dominated vaginal microflora[333, 411, 413]. Little is known concerning the biology and physiology of *L. gasseri* and thus the effects of acetate remain to be studied. In some Gram positive species such as *L. plantarum*, *L. sakei* and *L. rhamnosus,* acetate potentiated the expression of bacteriocin-encoding genes[565, 583-585] and thus one possibility is that it may have a similar effect in *L. gasseri*. Due to their small size and significant inter-species variation,

the annotation of bacteriocin-encoding genes is difficult[473, 586]. Recent genomic analysis of *L. gasseri* strains (including ATCC 33323) predicted that this species encodes acidocin A, gassericin and helveticin J[217-219]. However, in the reference strain only helveticin J, a class III bacteriocin that has been shown to have some activity against two clinical strains of *C. albicans* and *C. glabrata*[218], is represented in its genome. The fact that cellular density also affects the modulatory effect of acetate over bacteriocin-encoding genes[565, 585, 587] creates an important factor that has to be considered in a future study that may aim the study of the molecular mechanism underlying this acetate-induced virulence of *L. gasseri* against *Candida*.

III. Genomic expression profiling of *C. glabrata* while in co-cultivation with the vaginal species *L. gasseri* unveils aspects of this interaction and genes mediating competitiveness

Part of the results shown in this chapter are being prepared for publication:

Nuno A. Pedro, Sandra N Pinto, Sónia Silva, Nuno P Mira, Genomic expression profiling of *C. glabrata* while in co-cultivation with the vaginal species *L. gasseri* unveils aspects of this interaction and genes mediating competitiveness (submitted).

III.1. Abstract

Understanding how the interaction between *Candida* and lactobacilli is modulated can provide crucial insights to develop new anti-*Candida* based on probiotic strains. Thus, to get further insights about how *L. gasseri* and *C. glabrata* cells respond to the presence of each other, in this chapter we resorted to a transcriptomic analysis (based on dual RNA-sequencing) of a planktonic co-culture established between these different species. From this transcriptomic analysis we could detect 30 new transcripts that were not previously annotated in the genome of *L. gasseri* ATCC 33323, likely corresponding to novel genes. We could also identify what appears to be a large number of regulatory RNAs both in *L. gasseri* and in *C. glabrata*, including 38 and 42, respectively, that were differentially expressed in co-cultivation. After two hours of co-cultivation, 724 *C. glabrata* and 118 *L. gasseri* genes were found to differently expressed, compared to the levels they attained in the corresponding single-cultures. Examination of the transcriptomes of the *C. glabrata* and *L. gasseri* populations after 8h of co-cultivation revealed a differential expression of 629 *C. glabrata* and 178 *L. gasseri* genes. Among these, 40 and 590 *C. glabrata* genes were exclusively up- and down-regulated, respectively, after 2h of co-cultivation, while 38 and 39 *L. gasseri* genes were up- and down-regulated at the same time point, respectively. Inspection of the differentially expressed genes after 8h of co-cultivation revealed 397 and 138 *C. glabrata* genes to be up- and down-regulated, respectively, and 106 and 31 *L. gasseri* genes to be up- or -down-regulated, respectively. Interesting 8 and 64 *C. glabrata* genes were up- and down-regulated, respectively, and 19 and 17 *L. gasseri* genes were also up- and down-regulated in both growth stages thus suggesting a core response conserved during the entire co-cultivation period that was analysed. Functional clustering revealed that the *C. glabrata* or *L. gasseri* genes induced in the co-cultivation setting are involved in translation and ribosome biogenesis, in transport and metabolism of carbohydrate and amino acids, in transcription and in defense mechanisms (e.g., oxidative stress response, MDR and ABC transporters). It is, however, important to denote that for many of the DEGs both in *C. glabrata* and in *L. gasseri* could not be allocated to specific functional categories, likely reflecting the involvement in this interaction of genes that are yet still poorly characterized in the two species. Using the information gathered from the transcriptomic analysis it was examined the role in determining survival of *C. glabrata* in the presence of *L. gasseri* of the genes *CAGL0L03828g*, *CAGL0F04499g*, *CAGL0L10208g*, *RHR2* and *STR3*, found to be up-regulated in the co-culture setting at least in one timepoint. From these, only the deletion of *CAGL0L03828g*, encoding a protein involved in sterol biosynthetic process; of *CAGL0F04499g*, encoding a uridine transmembrane transporter; and of *CAGL0L10208g*, encoding a protein with phosphopantothenoylcysteine decarboxylase activity, significantly reduced *C. glabrata* growth in the presence of *L. gasseri*. The identification of these genes as determining competitiveness of *C. glabrata* while in the presence of *L. gasseri* is interesting as it may allow them to be used as therapeutic targets in screenings aiming to reduce performance of this pathogenic yeast in favour of the commensal microbiota.

III.2. Introduction

The burden posed by candidiasis (or infections caused by *Candida* spp.) is a pressing concern due to its high rates of morbidity (that result in prolonged hospital stays). Invasive candidiasis, involving colonization of the bloodstream or deep-seated organ infections, is life-threatening and has high rates of mortality (ranging between 20% to 75% of diagnosed cases, depending on the infecting species)[265-267, 514]. While invasive candidiasis is essentially observed in the immunosuppressed population, superficial infections, like vulvovaginal or oral candidiasis, are common even among the immunocompetent individuals[257, 268]. In fact, about 75% of women are estimated to experience vulvovaginal candidiasis (VVC) at least once during their lifetime, a substantial percentage (~138 million/year) suffer from recurrent infections, a condition known as recurrent vulvovaginal candidiasis (RVVC)[517, 588]. Although VVC is not lethal, its high incidence and, in some cases, high morbidity, has a high economic impact, estimated, in the US, in ~1.8 billions of dollars[517, 589]. *C. albicans* is the more common causative etiological agent in VVC responsible for ~85-90% of the cases[590, 591]. Recently, the incidence of vaginal candidiasis caused by non-albicans *Candida* species (or NACS) is increasing, a trend that has also been observed across the other forms of candidiasis[592-594]. Among NACS, *C. glabrata* is typically most prevalent, a trait that may result from the extreme genomic plasticity of this species and that prompts fast adaptive responses to the challenging environment of the different infection sites[257, 259].

Treatments currently available for VVC include various antifungals like nystatin, amphotericin B or azoles, with fluconazole and clotrimazole at the forefront of those more commonly used[595]. The efficacy of these therapeutic agents is being hampered by the persistent increase in the number of resistant *Candida* strains, this being particularly evident for azoles[596, 597] and for the *C. glabrata* species that is already “naturally” less susceptible to these antifungals[259]. In this context, alternative treatments to control VVC have been attempted including the use of lactobacilli-based probiotics[531, 598, 599]. In specific, the utilization of cocktails of *L. crispatus*, *L. gasseri*, *L. vaginalis*, *L. reuteri* and *L. rhamnosus* (alone or in consortia), alone or combination with other VVC therapies, have been explored in clinical trials[390, 530, 531, 600]. In general, improved control of VVC has been obtained exploring this avenue, however, different levels of effectiveness had been reported depending on predisposing factors of the subjects, the severity of the infection and also the lactobacilli strain used[369-377]. The choice to use lactobacilli stems from the long track record utilization of these species as probiotics (they are commonly used in multiple products aiming to restore the gastrointestinal microflora), but also because lactobacilli species are predominant in the vaginal microflora resulting in the idea that their abundance is linked to vaginal health[548, 601]. In specific, multiple metagenomic surveys undertaken with multiple cohorts of women showed that vaginal microbiomes are dominated by *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners*, with the abundance of each species depending on factors like age or race[22, 49, 93, 98]. Although a decrease in lactobacilli vaginal flora is clearly associated with the development of bacterial vaginosis[92, 93, 131], far less is understood

on whether these species also offer protection against VVC with some studies demonstrating a pattern of decreased lactobacilli abundance in women with VVC[139], while in others this is not observed[101, 102, 130, 136, 137, 140, 602]. More recently, it has been shown that the microbiome of women suffering from VVC have a similar abundance of lactobacilli than healthy ones, however, the predominant species in the VVC cohort was *L. iners*, while in the others *L. crispatus* or *L. gasseri* were dominant[101, 130, 140]. It is thus likely that the equilibrium established between the different indigenous lactobacilli species may determine a more or less favourable environment for yeasts to thrive[134].

Although *in vivo* the role of lactobacilli in protecting against VVC remains elusive, *in vitro* it has been clearly shown that they can inhibit growth and virulence traits of *Candida*[390, 394, 529]. Historically this inhibitory effect has been linked with the maintenance of an acid vaginal pH (due to increased production of lactic acid[85]), however, at the typical vaginal pH (close to 4) the majority of this acid will prevail in its non-toxic (and even metabolizable by *Candida*[433, 434]) lactate form. In fact, concentrations of about 160mM lactic acid were ineffective in exerting inhibition against *Candida in vitro*([430]) which far exceed those that usually are observed to occur in the vaginal tract[411, 413] and metabolomics studies could not found a correlation between the amounts of lactic acid and the anti-*Candida* effect prompted by supernatant of vaginal lactobacilli species[390]. It thus seems that the anti-*Candida* effect of lactobacilli goes beyond the mere production of lactic acid. More recently, the inhibitory effect of *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*) over *Candida* was associated to the production of 1-acetyl-β-carboline[395], however, the underlying biosynthetic production pathway was not elucidated and thus it is not known whether indigenous vaginal species are also capable of producing it. Phenyllactic acid[399], mevalonolactone[401], 2-hydroxyisocaproic acid (HICA)[402, 403], 3-hydroxyoctanoate[405] are examples of other metabolites produced by lactobacilli that also have a described anti-*Candida* potential, however, their production pathways are not well characterized and, thus, it is not known whether vaginal species produce it. The production of biosurfactants as a mean to reduce adhesiveness of *Candida* to the epithelial cells[409, 603, 604] and the production of bacteriocins or of H_2O_2 have also been suggested to underlie the antagonistic effects of lactobacilli, however, these molecular mechanisms had not been studied in the specific context of interactions with *Candida* cells.

Recently a study from our laboratory showed that the vaginal species *L. gasseri* inhibits growth and promotes decreased viability of *C. albicans* and *C. glabrata* while in co-culture, both in planktonic and in mixed biofilms[605]. To get further insights into this, we have used the same experimental setting to undertake, for the first time, a transcriptome analysis of a *L. gasseri-C. glabrata* co-culture using dual RNA-sequencing. Other transcriptomic analyses have been undertaken to study *C. albicans* and lactobacilli interaction exposing the yeast cells to *L. rhamnosus*[398] and *L. crispatus*[140] in co-infection of intestinal epithelial cells (tri-culture) and in biofilm forming conditions, respectively. These works revealed that the co-cultivation caused profound alternations in the *C. albicans* metabolism that resulted in a slower growth of the yeasts due to exhaustion of the most favourable carbon and nitrogen sources from the medium[140, 398],

this being accompanied by an attenuation of virulence[140, 398]. This translated into an overexpression of *Candida* genes required for amino acid uptake, while the expression of genes involved in glycolysis decreased[398]. Genes encoding enzymes of the oxidative phosphorylation pathway were also found to be down-regulated along co-cultivation with *L. rhamnosus*, this being believed to promote a reduction in ATP synthesis and, consequently, reduced fungal growth, filamentation, and virulence[606]. Co-cultivation with *L. rhamnosus* has also been found to reduce the expression of the tyrosine phosphatase *PTP3*, encoding a protein required for hyphal maintenance[607]; of the transcription factor *AHR1* that regulates the virulence genes *ALS3* and *ECE168*; and of Orf19.4292 that encodes the prevacuolar trafficking protein Pep12, an essential determinant for virulence in mice[608]. These observations suggested that besides the effects on metabolism, the presence of *L. rhamnosus* also caused direct modifications in virulence genes, remaining to be clarified whether these stemmed from the metabolic alterations or had another origin.

Different from what has been done in most studies that focused only on the yeast transcriptional modifications[140, 398], herein the transcriptional changes of *L. gasseri* are also considered resulting in a dual RNA-sequencing analysis of the co-cultivation. Additionally, to our best knowledge, this is the first focused on the transcriptomics response of the species *C. glabrata* to the presence of lactobacilli. It is expected that the identification of the molecular players modulating the interaction between *L. gasseri* and *C. glabrata* can also enlighten important aspects of the way in which these species interact with each other. Such knowledge could be leveraged to develop better therapeutics that may tip the ecological vaginal balance in favour of the bacteria, thus providing alternatives to currently used antifungal molecules for the treatment of vaginal candidiasis.

III.3. Materials and methods

III.3.1. Strains and growth media

For the transcriptomic profiling were used the strains *L. gasseri* ATCC 33323 (acquired from DSMZ) and *C. glabrata* KUE100, a wild-type strain derived from the CBS138 strain[433]. A set of *C. glabrata* single mutants (kindly provided by Dr Hiroji Chibana, from the Medical Mycology Research Center at Chiba University, Japan), built in the background of KUE100, that included Cg∆*CAGL0L03828g*, Cg∆*CAGL0F04499g*, Cg∆*CAGL0L10208g*, Cg∆*RHR2* and Cg∆*STR3* were also used. The MRS medium used to co-cultivate yeasts and bacteria contains, per liter, 10 g casein peptone (Gibco); 10 g meat extract (Panreac AppliChem); 5 g yeast extract (Gibco); 20 g glucose (Nzytech); 1 g Tween 80 (Sigma); 2 g K_2HPO_4 (Merck); 5 g sodium acetate (Merck); 3 g ammonium sulphate (Panreac AppliChem); 0.20 g $MgSO_4.7H_2O$ (Labchem) and 0.05 g $MnSO_4.H_2O$ (Sigma). After preparation, the pH of MRS was adjusted to 6.2-6.5 using HCl or NaOH. Solid YPD medium was used for maintenance of the yeast strains, and contained, per liter, 20 g glucose (Nzytech), 20 g peptone (Gibco) and 10 g yeast extract (Gibco) while the bacterial strain was kept at -80ºC in MRS and 86% glycerol. Solid YPD or MRS were prepared by supplementing the corresponding liquid medium with 2% and 1.5% agar (Nzytech), respectively. Media were prepared using deionized water and sterilized by autoclaving for 15 min at 121°C and 1 atm.

III.3.2. Co-cultivation in liquid MRS medium of *L. gasseri* and *C. glabrata*

To examine growth of *L. gasseri* and of *C. glabrata* KUE100 or of the deletion mutants Cg∆*CAGL0L03828g*, Cg∆*CAGL0F04499g*, Cg∆*CAGL0L10208g*, Cg∆*RHR2* and Cg∆*STR3* in liquid MRS, a pre-inoculum of each strain was prepared, in MRS. The cells were then cultivated, overnight, at 37ºC with an orbital agitation of 100 rpm. On the next day, these cells were used to inoculate (at an initial OD_{600nm} of 0.4 for *L. gasseri* and 0.1 for *C. glabrata*) fresh MRS medium, after which the cultures were cultivated at 37ºC and 100 rpm for 96 hours. Viability of the yeasts and of the bacteria in single- or in co-cultivation was followed based on the number of colony-forming units (CFUs). For this, aliquots of co-cultures were taken, serially diluted, and plated on MRS supplemented with 96 mg/L fluconazole (an antifungal concentration that fully prevented growth of *Candida* colonies and thus only *L. gasseri* colonies were visible) or in YPD supplemented with 300 mg/L tetracycline (an antibiotic concentration that fully prevented the growth of *L. gasseri* colonies and therefore only *Candida* colonies were visible). The number of *Candida* colonies formed onto the surface of YPD plates was counted after 2 days of incubation at 30ºC, while the number of *L. gasseri* colonies formed onto the surface of MRS plates was counted after 2 days of plate incubation at 37ºC in a Genbox (Biomerieux) with a candle inside to assure microaerophilia[541, 542].

III.3.3. Transcriptomic analysis

For the transcriptomic analysis, the single and co-cultures of *L. gasseri* ATCCC 33323 and *C. glabrata* KUE100 were cultivated, in triplicates, in 120 mL of MRS medium, at 37ºC and 100 rpm, in shake-flasks, as detailed above. After 2 and 8 hours of single or co-cultivation, cells were harvested, centrifuged (at 6000 rpm for 6 minutes at 4ºC) and stored at -80ºC until further use. RNA extraction was performed using the RiboPure™ RNA Isolation Kit (Ambion, Life Technologies, CA) and following the manufacturer's instructions. Quality and integrity of the purified RNA were determined using a fragment analyser at Instituto Gulbenkian de Ciência in Oeiras, Portugal. The three replicates obtained from *L. gasseri* and *C. glabrata* single and co-cultures were pooled together into 6 RNA pools. To prepare these pools all RNA samples were quantified, pooled at equimolar concentrations and sent to CD Genomics (Ramsey Road, Shirley, New York, USA) for sequencing. Briefly, sequencing was performed using Illumina HiSeq PE150 generating a total of 20M reads (6.0Gbase data) per sample. Before mapping, a double rRNA depletion protocol was undertaken in the samples using the Ribo-Zero Magnetic Gold epidemiology kit (Illumina) according with the manufacturer's guidelines[609]. For cDNA library construction, the RNA samples were fragmented using ultrasound (four pulses of each 30s) at 4ºC to generate ~200–400 nt (average) fragmentation products. Short RNA fragments (<20 nt) were removed using the Agencourt RNAClean XP kit (Beckman Coulter Genomics). The remaining fragments were dephosphorylated with Antarctic Phosphatase (NEB), re-phosphorylated with T4 Polynucleotide Kinase (NEB), and poly(A)-tailed using poly(A) polymerase (NEB). An RNA adapter was ligated to the 5′ monophosphate of the RNA fragments using T4 RNA ligase (NEB). First-strand cDNA synthesis was performed using an oligo(dT) adapter primer and the M-MuLV reverse transcriptase (NEB). The resulting cDNAs were PCR-amplified to about 10–20 ng/μL using the Phusion high fidelity DNA polymerase (NEB) and primers designed for TruSeq sequencing according to the instructions of Illumina. The resulting cDNA libraries were purified using the Agencourt AMPure XP kit and analysed by capillary electrophoresis (Shimadzu MultiNA microchip electrophoresis system)[609]. After rRNA depletion, library construction and sequencing, the reads were mapped to the respective reference genome. Mapping of the sequenced reads was performed against the genomes of *L. gasseri* ATCC 33323 (extracted from NCBI: Bioproject - PRJNA84) and *C. glabrata* CBS138 (extracted from the *Candida* Genome Database). Expression levels were measured as "FPKM", normalizing to the total number of mapped reads (Fragments Per Kilobase of transcript per Million mapped reads)[610]. To analyse gene expression HTSeq[611] with model intersection-nonempty was used. Automatic detection of novel transcripts was performed using Rockhopper[612] software, followed by manual curation. For gene differential expression analysis was used EBSeq[613]. All required in-house bioinformatic analyses were performed using QIAGEN CLC Genomics Workbench 10.1.1.

III.4. Results

III.4.1. Overview of the RNA sequencing data unveils novel aspects of the functional analysis of *L. gasseri* and *C. glabrata* genomes

To get a glimpse of the molecular changes occurring in the transcriptome of *C. glabrata* and *L. gasseri* during planktonic single and co-cultivation in MRS medium, an experimental setup similar to the one explored in Chapter II was used. Briefly, *C. glabrata* and *L. gasseri* were cultivated using a yeast:bacteria proportion of 1:4, at 100 rpm and 37ºC and after 2 and 8 hours they were collected for transcriptome analysis. These two time-points correspond, respectively, to the early period of adaptation of each species to the co-cultivation setting, or to the entrance in stationary phase (Figure III.1). In both these two time points the viability of the yeasts in the co-culture setting was not significantly different than the one registered in single-culture (Figure III.1), however, had we opted for a later period in which reduced viability of *C. glabrata* cells was observed, we could get a strong transcriptional responses caused by the cell death thus masking the more specific response triggered by the yeast cells in response to the bacteria.

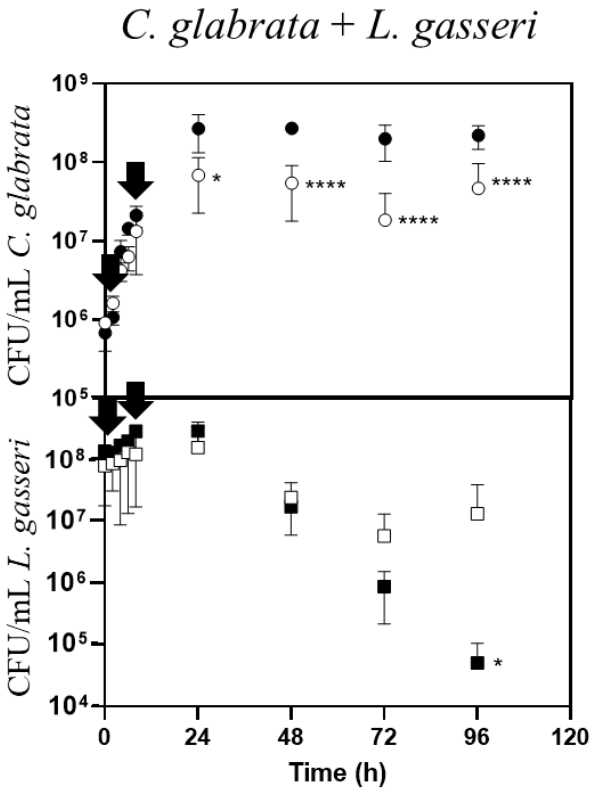


Figure III.1. Cellular viability of *C. glabrata* and *L. gasseri* along single or co-cultivation in MRS medium. After inoculation, cells of *C. glabrata* (○,●) and *L. gasseri* (□,■) were cultivated at 37ºC and 100 with the growth of the different species being accompanied based on cellular viability, as detailed in materials and methods. For total RNA extraction cells from single and co-cultures were collected at the time points highlighted by the black arrows (2 and 8 hours growth). Filled symbols correspond to the samples taken during single-species cultivation while open symbols correspond to the samples taken during co-cultivation. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

The reads obtained after the RNA-seq step were mapped to the *L. gasseri* and *C. glabrata* genomes resulting in the data shown in Annex Table III.1. As expected, the reads obtained from the single-cultures mapped at high percentages (ranging between 96%-98%) to the reference genomes of *C. glabrata* and *L. gasseri*. In the co-cultures the reads obtained at 2h of co-cultivation distributed between *L. gasseri* and *C. glabrata* in a relatively equivalent manner (~40% for each species), while at the 8h period there was a predominance of reads gathered from *C. glabrata* (~70% of the overall), compared to those gathered from *L. gasseri* (that were on the range of 20-30%). To the best of our knowledge, the current study is the first undertaking a transcriptomic analysis of *L. gasseri* for which we have also taken a closer look into the mapping results, hoping to further contribute for the functional analysis of the genome of the ATCC 33323 strain. From this more thorough analysis we could detect 30 transcripts (produced both in single and in co-cultures) that map in regions not previously annotated as genes in the genome of *L. gasseri* ATCC 33323 (Table III.1 and in more detail in Annex Table III.2). These hypothesized new genes were subjected to a BlastX analysis against the general database of proteins deposited at NCBI, revealing that some bear similarity (albeit at different extents) to proteins annotated in the genome of other *L. gasseri* strains or of species closely related to *L. gasseri* (see Table III.1). This is, for example, the case of genes 2 and 4 found to bear similarity against proteins WP_223891855.1 and WP_015981461.1 proteins from *L. paragasseri*, or of gene 1 that had been annotated in the genome of the *L. gasseri* strain MV-22 (Table III.1 and Annex Table III.2). For 16 of the hypothesized new *L. gasseri* transcripts (from 15-30), we could not detect significant homologies with described proteins (Annex Table III.2). Despite the identified similarities, the corresponding homologous proteins to these new hypothesized “new” genes identified in *L. gasseri* ATCC 33323 do not have a known function and thus it is hard to predict what can be the biological role of these proteins. The exception to this are gene 2, similar to a prenyltransferase that are cell wall-anchored surface proteins used as signalling peptides; and gene 1 predicted to be a signal peptide protein from the YSIRK family involved in peptide signalling. Notably, we could also identify two transcripts mapped in regions of the *C. glabrata* CBS138 genome that had no annotations for genes, CgNovel_870 and CgNovel_1656, although these bear similarities with two hypothetical proteins KAI8395120.1 and KAH7579243.1, annotated in the genome of *C. glabrata* P35-2 and CAS08-0016 strains, respectively.

Table III.1. Potential new transcripts of *L. gasseri*. Transcripts were identified manually by the presence of reads in genomic regions without a described annotation. Analysis was performed using CLC Genomics Workbench 10.1.1. Blastx analysis was performed in NCBI. Proteins were considered similar when the BlastX had a p-value under 10^{-15}.

Candidate gene	Genome location	Blastx best hit	Blastx best hit description	P-value
1	157047	EFQ46842.1	Gram-positive signal peptide protein, YSIRK family [*Lactobacillus gasseri* MV-22]	0
2	276100	WP_223891855.1	prenyltransferase [*Lactobacillus paragasseri*]	3.00E-23
3	274034	ABJ59649.1	hypothetical protein LGAS_0240 [*Lactobacillus* gasseri]	2.00E-36
4	612818	WP_015981461.1	hypothetical protein [*Lactobacillus paragasseri*]	5.00E-48
5	1479598	DAW44901.1	hypothetical protein [*Caudoviricetes* spp.]	1.00E-19
6	1567401	WP_230577983.1	hypothetical protein, partial [*Lactobacillus gasseri*]	1.00E-32
7	1810871	WP_221930141.1	hypothetical protein, partial [*Lactobacillus gasseri*]	2.00E-16
8	477048	WP_230577983.1	hypothetical protein, partial [*Lactobacillus gasseri*]	9.00E-35
9	535335	EFQ46544.1	hypothetical protein LBGG_02068 [*Lactobacillus gasseri* MV-22]	7.00E-25
10	976092	EEQ25408.1	hypothetical protein HMPREF0890_0084 [*Lactobacillus gasseri* 202-4]	4.00E-19
11	1793776	WP_230577983.1	hypothetical protein, partial [*Lactobacillus gasseri*]	1.00E-26
12	1817748	WP_003648189.1	hypothetical protein [*Lactobacillus gasseri*]	1.00E-49
13	1560729	WP_222423892.1	hypothetical protein, partial [*Lactobacillus gasseri*]	7.00E-29
14	652903	WP_015981461.1	hypothetical protein [*Lactobacillus paragasseri*]	8.00E-49

Another interesting finding that we have obtained upon a more exhaustive analysis of the read mapping was the identification of transcripts, both from *L. gasseri* and *C. glabrata*, mapping in the opposite strand where are annotated genes, in most cases these transcripts encompassing only part of the gene, as detailed in (Annex Table III.3). With the data that we have available, it is not possible to determine whether these transcripts correspond to regulatory non-coding RNAs or whether they result from artefactual anti-sense transcripts produced along the preparation of the libraries, as before described to occur[614, 615]. Nonetheless, it is interesting that 157 of these *C. glabrata* transcripts herein detected had been previously detected in a study that specifically searched for regulatory RNAs in this pathogenic yeast species (Annex Table III.3)[616]. Although the existence of regulatory RNAs has not been specifically described in *L. gasseri*, it has been reported in *L. casei* and in *L. salivarius*[617, 618]. Notably, the structure of these putative anti-sense RNAs appears to be quite divergent with some comprising the entire gene size while others are bigger than the gene (both from the 5' and 3' termini)(as detailed in Annex Table III.3). It was also noticeable in *L. gasseri* some anti-sense RNAs mapping in more

than one gene (e.g., LgNovel_74) (Annex Table III.3) or in intergenic regions (as it was the case of the *C. glabrata* antisense Novel_1550, Novel_1567 and Novel_1278.). The co-cultivation affected the expression of these presumed anti-sense RNAs in both yeast and bacteria, with 42 of these being differently expressed in *C. glabrata* (e.g., Novel_973 and Novel_1175 up-regulated after 8h) and 38 in *L. gasseri* (e.g., Novel_77 and Novel_119 up-regulated after 8h), as detailed in Annex Table III.3.

III.4.1.1. Transcriptomic alterations of *C. glabrata* and *L. gasseri* along co-cultivation

The co-cultivation setting induced prominent alterations in the transcriptome of both *L. gasseri* and *C. glabrata*, as depicted by the results shown in Figure III.2 and the volcano plots depicted in Figure III.3 panel A and Annex Figure III.1). In total, around 1300 *C. glabrata* genes (detailed in Annex Table III.4) changed their expression during co-culture with *L. gasseri*, with the majority of the changes being observed after 2 hours, although the difference is small (as detailed in Figure III.2). Notably, most the *C. glabrata* differentially expressed genes at 2 hours of co-cultivation were down-regulated, while after 8 hours the number of up-regulated genes surpassed those repressed by the presence of *L. gasseri* (Figure III.2). This time-dependent effect observed in *C. glabrata* genomic expression may reflect the accumulation in the broth of metabolic products produced by the bacteria, like lactic acid, that may exert differential effects in the yeast cells. In the case of *L. gasseri*, the transcriptome-wide modifications were much milder with approximately 300 genes being differentially expressed in the two times of the co-culture (Figure III.2 and detailed in Annex Table III.4), suggesting that the presence of the bacteria is more impactful for the yeast cells than the other way around. Clustering based on gene expression of the different samples, shown in Figure III.3 panel B, also clearly shows that cultivation time has a significant impact in the transcriptomes of both species (note the similarity of patterns observed for the transcriptomes obtained after 2 and 8h in single species cultivation), with a stronger impact for *C. glabrata*.

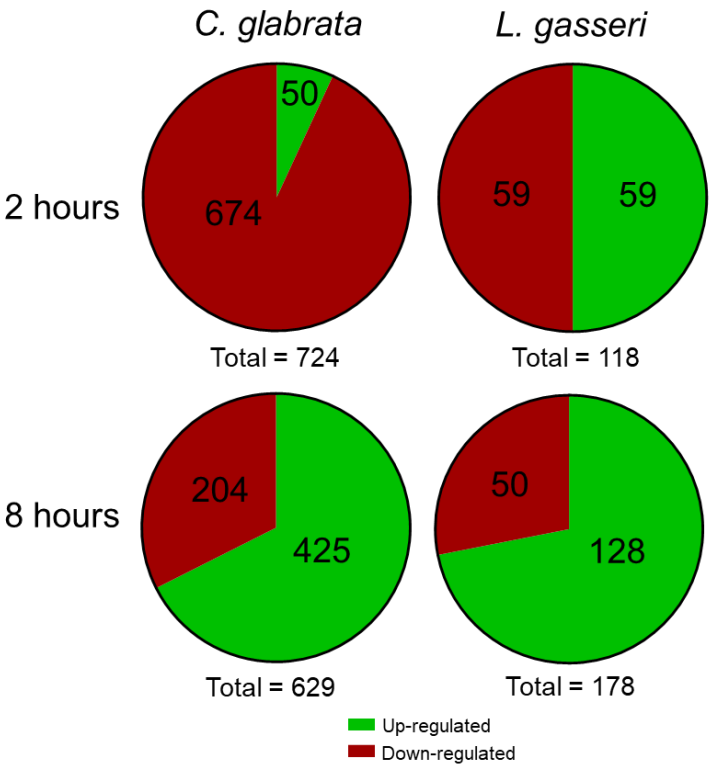


Figure III.2. Differentially expressed genes of *C. glabrata* and *L. gasseri* at 2 and 8 hours of growth at 37ºC and 100 rpm between single and co-cultivation. Gene differential expression was determined using the single-culture as control.

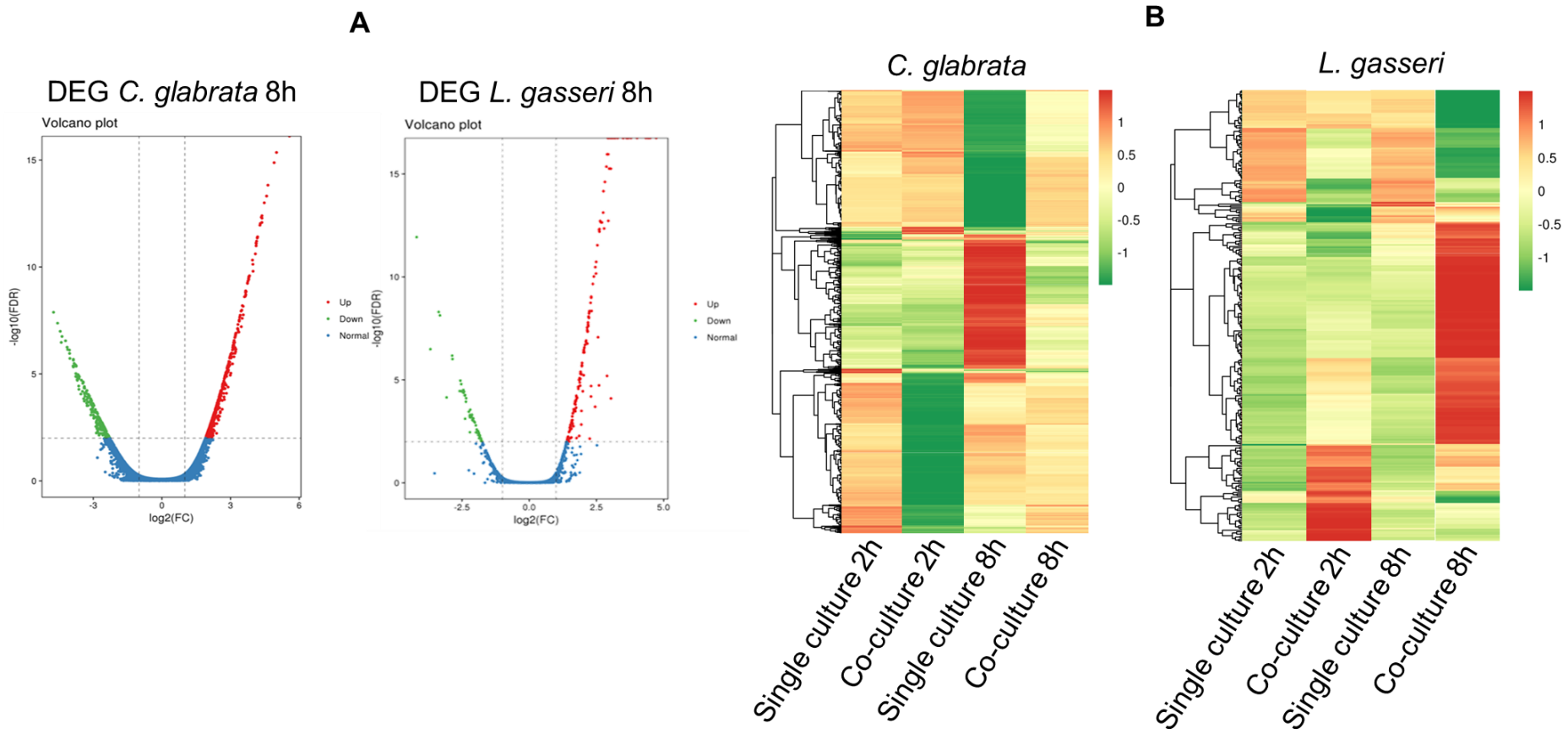
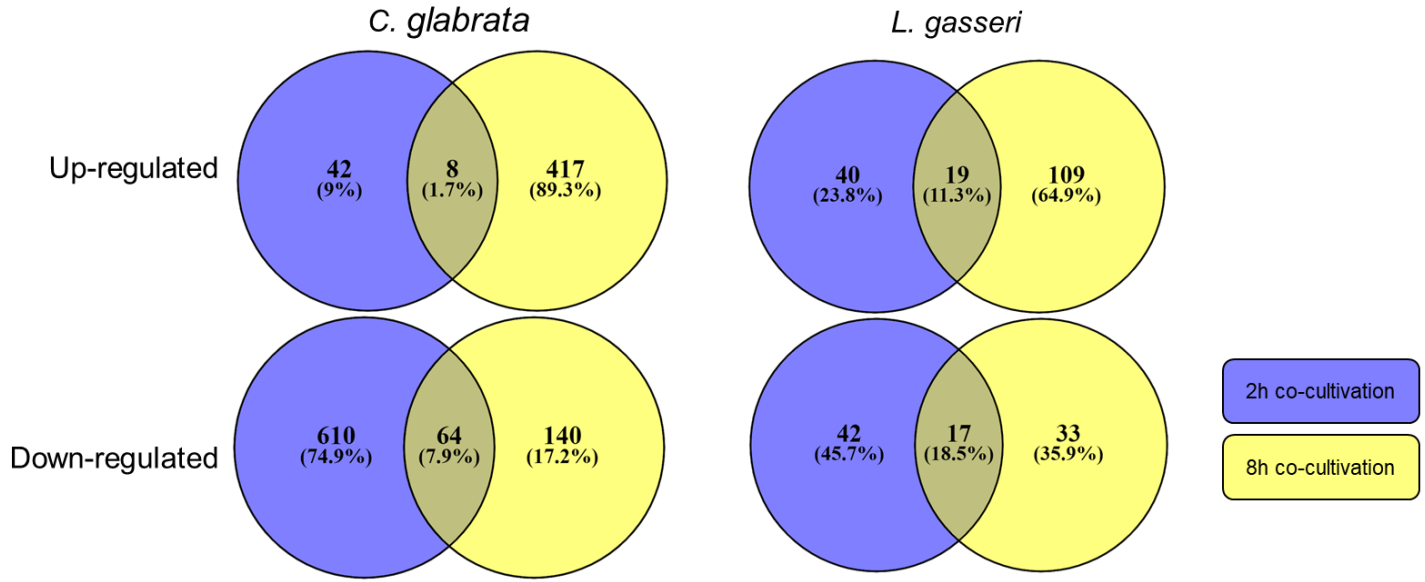


Figure III.3. (A) Volcano plots of the differentially expressed genes (log2(FC)) of *C. glabrata* and *L. gasseri* after 8h of growth in MRS at 37ºC and 100 rpm. Each dot represents a single gene and the red dots are the up-regulated genes while the green dots are the down-regulated genes in co-cultivation. Blue dots were not significantly differentially expressed in co-cultivation settings. **(B)** Cluster analysis of the samples genomic expression. Clustering was performed based on FPKMs, where log2(FPKM) was used. The red colour represents genes with higher expression, while the green colour represents genes with lower expression. Different columns represent different samples, while different rows represent different genes.

Comparison of the individual sets of genes considered to be up- or down-regulated at the early or later time points of the co-culture resulted in the Venn diagrams shown in Figure III.4. From that analysis, it was evident that the number of *C. glabrata* and *L. gasseri* genes that are maintained differently expressed at both time points is relatively small suggesting that cells trigger short- and later- responses that do not appear to make use of the same players. In Figure III.5 it is depicted, in more detail, the pattern of expression of those genes that were found to be up- or down-regulated in the co-culture at both time points, along with their biological function. The 8 *C. glabrata* genes up-regulated on both time points showed higher activation at the later-time point and comprised: *CgTRR2* and *CgTSA1*, encoding two enzymes involved in antioxidant responses; *CgTIR2* and *CgAWP3*, encoding two cell wall proteins; *CAGL0C03740g* and *CAGL0L06930g*, encoding proteins of unclear function; *CAGL0L08114g* encoding a structural constituent of ribosomes; and *CAGL0J04576g* encoding a protein involved in synthesis of diphthamide. Among the 64 genes of *C. glabrata* that were simultaneously down-regulated at 2 and 8h of the co-cultivation were the hexose transporters *HXT5* and *HXT4/6/7*; two genes responsible for glycerol metabolism, *CAGL0E03916g* and *CAGL0H06699g*; *CAGL0M00550g* and *CAR2* involved in cysteine and arginine metabolism), and 18 genes with unclear biological function in this yeast (Figure III.5 and Annex Table III.5). In the case of the 19 *L. gasseri* up-regulated genes in both time points, 5 showed a lower pattern of induction at 8h (*LGAS_RS08210*, *LGAS_RS00915*, *LGAS_RS00910*, *LGAS_RS05255* and *LGAS_RS02640*) while the remaining exhibited higher levels of up-regulation at the later time point. These up-regulated genes include mostly transporters involved in transport of glycerol (*LGAS_RS01070*), water (*LGAS_RS02355*, a predicted aquaporin) and the PTS glucose transporters *LGAS_RS08210* and *LGAS_RS02640* (Figure III.5 and Annex Table III.5). All the down-regulated genes of *L. gasseri* at 2h and 8h of co-cultivation showed higher levels of repression at the later time point (see Figure III.5 and Annex Table III.5).



Up-regulated at 2h	e.g., *AWP6* (adhesion); *CTA1* (OSR); *CAGL0F04499g* (aa metabolism)	Up-regulated at 2h	e.g., *LGAS_RS02540* (carbon uptake); *LGAS_RS01120* (aa permease); *LGAS_RS06995* (MFS transporter)
Up-regulated at 8h	e.g., *HXT1* and *HXK2* (carbon metabolism); *ARG8* (aa metabolism); *EPA1* (adhesion)	Up-regulated at 8h	e.g., *LGAS_RS08770* and *LGAS_RS04265* (aa permease); *LGAS_RS01085* and *LGAS_RS01080* (sugar ABC transporter)
Up-regulated at 2h and 8h	e.g., *AWP3* (adhesion); *TRR2* and *TSA1* (OSR)	Up-regulated at 2h and 8h	e.g., *LGAS_RS08210*, *LGAS_RS01065* and *LGAS_RS02630* (carbon uptake and metabolism); *LGAS_RS03610* (MFS transporter)
Down-regulated at 2h	e.g., *PYK1* and *GND1* (carbon metabolism); *ERG5* and *ERG9* (cell membrane biosynthesis)	Down-regulated at 2h	e.g., *LGAS_RS02745* (carbon metabolism); *LGAS_RS00155* (magnesium transporter); *LGAS_RS04925* (transcriptional regulator)
Down-regulated at 8h	e.g., *AWP1* (adhesion); *PGM1 and CAGL0E05610g* (carbon metabolism)	Down-regulated at 8h	e.g., *LGAS_RS01000* (magnesium transport); *LGAS_RS08335* (ABC transporter); *LGAS_RS00390* (glycosyltransferase)
Down-regulated at 2h and 8h	e.g., *HXT5* and *HXT4/6/7* (carbon metabolism); *CAGL0B04213g* and *CAGL0E03916g* (glycerol metabolism); *CAGL0M00550g* (aa metabolism)	Down-regulated at 2h and 8h	e.g., *LGAS_RS07640* and *LGAS_RS00125* (energy conversion); *LGAS_RS02335* (helveticin)

Figure III.4. Venn diagram of the up-regulated and down-regulated genes of *C. glabrata* and *L. gasseri* at 2 and 8 hours of growth in co-cultivation. Growth was performed in MRS at 37ºC and 100 rpm. In blue is represented the DEG of both species after 2h of co-cultivation, while in yellow is represented the DEG after 8h of co-cultivation. Intersections represent genes that are simultaneously up-regulated or down-regulated at different growth stages. Diagram was made using Venny 2.1.0. The list of genes used to construct the Venn diagrams is in Annex Table III.4. Abbreviations: aa – amino acid; OSR – oxidative stress response; MFS – major facilitator superfamily; ABC – ATP-binding cassette.

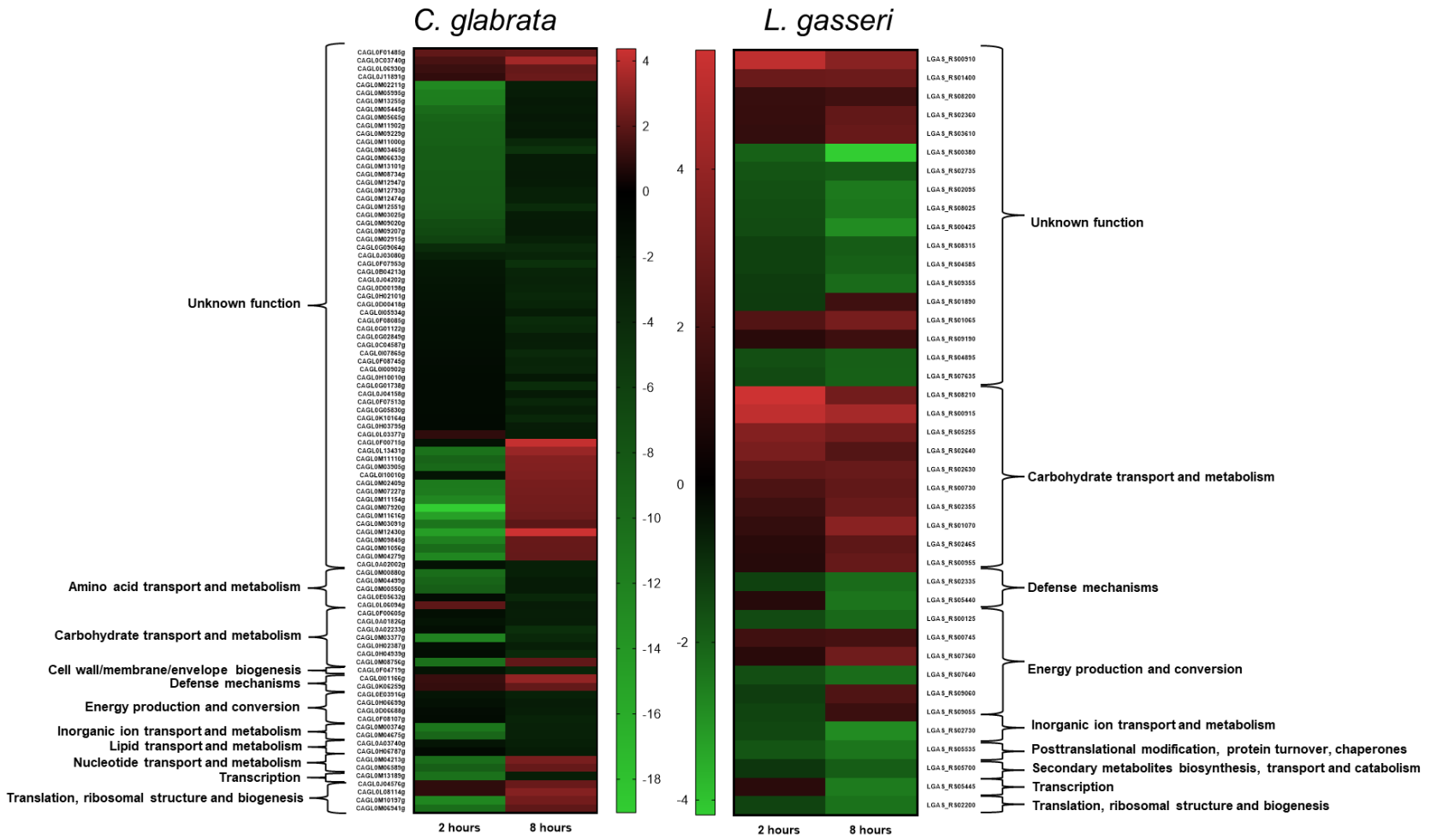


Figure III.5. Heatmap of the DEGs of *C. glabrata* and *L. gasseri* that were simultaneously identified at both cultivation time points. Green genes were found to have a negative fold-change (down-regulated) while red genes have a positive fold-change (up-regulated). Note that some genes are up or down-regulated at both culture stages, while some genes were up-regulated at 2 hours and down-regulated at 8 hours and vice-versa. COGs of each gene are also indicated. The list of genes used to construct the heat map is in Annex Table III.5.

III.4.2. Function analysis of the differential expressed genes in the co-cultivation

To get further insights into the biological function of the *C. glabrata* and *L. gasseri* genes differently expressed upon the co-cultivation a functional clustering was performed based on COG assignment. The results obtained are shown in Figure III.6. Notably, many of the DEGs of *C. glabrata* (of about 70%) and *L. gasseri* (of about 45%) could not be assigned to any COG annotation suggesting the involvement in this response of many genes that are, as of yet, poorly characterized. Indeed, *CAGL0B00792g*, *CAGL0A02255g*, *CAGL0E05522g*, *CAGL0E03498g* and *CAGL0K07205g* were between the most significantly *C. glabrata* up-regulated genes in the presence of *L. gasseri*, all of these encoding proteins with a very poorly characterized functions (Annex Table III.4). This also reflects some inaccuracy of the automatic COG functional assignment since in some cases the genes without COG class do have functions that are relatively well characterized as it is the case of the *CgAWP3*, *CgAWP6* and *CgEPA1*, all predicted to encode *C. glabrata* adhesins and well characterized for their involvement in the ability of the cells to adhere to both biotic and abiotic surfaces[619] and found to be up-regulated in the co-culture setting. In the case of *L. gasseri*, this lack of functional associations may result from the generalized poor knowledge that we still have about this species, that consequently results in limited functional analyses for most genes. Among the identifiable COG annotations, “translation”, “ribosomal structure and biogenesis”, “carbohydrate transport and metabolism”, “amino acid transport and metabolism”, “transcription” and “defense mechanisms” were the classes encompassing a higher number of DEGs in the two species (Figure III.6). In general, the functional classes encompassing more DEGs were identical in the two species, with the exception of the “translation, ribosomal structure and biogenesis” functional class that was only relevant in the dataset of *C. glabrata* DEGs but not in *L. gasseri*. In the following sections, we will provide a more comprehensive view of the DEGs included in these different functional classes and that help to better understand the yeast-bacteria interaction.

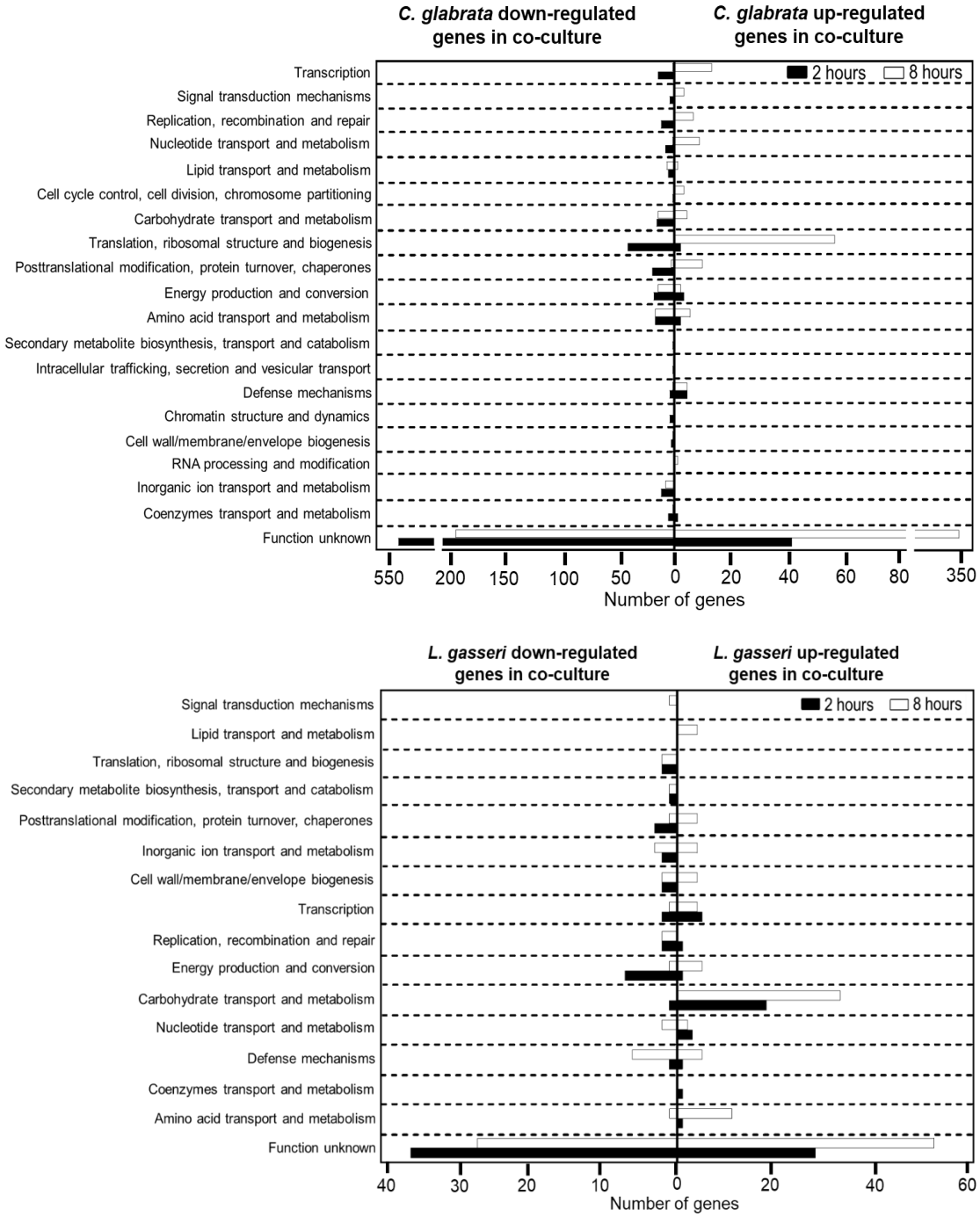


Figure III.6. Cluster of orthologous groups (COGs) of the DEGs at 2 and 8 hours of *C. glabrata* and *L. gasseri* in co-cultivation compared with the respective single-cultures. Among the genes that were classified, most genes of *C. glabrata* were attributed to translational, ribosomal structure and biogenesis while most genes of *L. gasseri* were attributed to carbohydrate transport and metabolism. However, for many of the DEGs was not possible to determine their COG annotation (Function Unknown). COG classification for each gene is given in Annex Table III.6.

Carbohydrate metabolism and transport

The "carbohydrate metabolism and transport" was the COG class having a higher number of *L. gasseri* DEGs both at the early and late phase of the co-cultivation (including 18 and 33 genes up-regulated, respectively) (Figure III.6). In specific, it was observed the up-regulation of the predicted PTS transporters *LGAS_RS02640*, *LGAS_RS08210*, *LGAS_RS00915*,

LGAS_RS00730; the sugar ABC transporters *LGAS_RS01085* and *LGAS_RS01080*, phospho-beta-glucosidase (*LGAS_RS00905*) and of the family 1 glycosyl hydrolase *LGAS_RS00930*, required for hydrolysis of the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Table III.2). This relatively high number of up-regulated genes involved in the transport of carbohydrates upon cultivation of *L. gasseri* with *C. glabrata* suggests competition of the two species for the sugar available, which is relatively expected. In contrast to *L. gasseri*, in *C. glabrata* most genes clustered in the "carbohydrate transport and metabolism" class were down-regulated in co-cultivation (Figure III.6). These included, for example, the pyruvate kinases *PYK1* and *CAGL0E05610g* and the trehalose and fructose phosphatases *CAGL0H02387g* and *CAGL0H04939g* (Table III.3). Additionally, hexose transporters *HXT5* and *HXT4/6/7*, were also down-regulated at both time points of the co-culture (Table III.3). Interestingly, genes *CYB2* and *DLD1*, involved in the utilization of lactate were found to be up-regulated at 2h growth in *C. glabrata* (Annex Table III.4), which is coherent with the production of lactic acid by lactobacilli which the cells could be using as a carbon source as previously described[433, 434], however these were not differentially expressed after 8h of growth.

Table III.2. *L. gasseri* up-regulated genes belonging to the COG carbohydrate metabolism and transport. Table describes the gene ID, the log2 foldchange (Log2FC)) after 2h and 8h and a brief description of the gene.

Gene ID	2 hours Log2FC	8 hours Log2FC	Description
LGAS_RS05255	3.52	3.04	RpiB/LacA/LacB family sugar-phosphate isomerase
LGAS_RS02465	1.08	2.49	PTS transporter subunit EIIC
LGAS_RS00730	2.02	2.60	PTS sugar transporter subunit IIA
LGAS_RS02630	2.63	2.76	alpha,alpha-phosphotrehalase
LGAS_RS02355	1.66	2.78	aquaporin family protein
LGAS_RS01070	1.37	3.66	sn-glycerol-3-phosphate ABC transporter ATP-binding protein UgpC
LGAS_RS00955	1.01	2.70	PTS sugar transporter subunit IIC
LGAS_RS00915	5.12	4.41	PTS cellobiose transporter subunit IIC
LGAS_RS02640	3.25	2.21	PTS glucose transporter subunit IIA
LGAS_RS08210	5.51	3.06	PTS glucose transporter subunit IIA
LGAS_RS01060	1.43	-	glycoside hydrolase family 65 protein
LGAS_RS00725	1.07	-	1-phosphofructokinase
LGAS_RS02440	1.24	-	PTS fructose transporter subunit IIB
LGAS_RS01050	1.12	-	alpha-glucosidase
LGAS_RS08625	1.35	-	PTS beta-glucoside transporter subunit EIIBCA
LGAS_RS02540	1.39	-	PTS sugar transporter subunit IIC
LGAS_RS01090	1.25	-	alpha-glucosidase
LGAS_RS00960	1.22	-	glycoside hydrolase family 1 protein
LGAS_RS00570	-	1.87	PTS sugar transporter subunit IIB
LGAS_RS08245	-	1.45	PTS sugar transporter subunit IIC
LGAS_RS00580	-	2.31	PTS system mannose/fructose/sorbose family transporter subunit IID
LGAS_RS00840	-	1.52	sugar transporter
LGAS_RS08740	-	3.02	PTS sucrose transporter subunit IIABC
LGAS_RS02810	-	1.48	PTS sugar transporter subunit IIC
LGAS_RS08035	-	1.90	N-acetylglucosamine kinase
LGAS_RS09105	-	1.86	PTS sugar transporter subunit IIC
LGAS_RS08485	-	1.59	1-phosphofructokinase
LGAS_RS08815	-	1.61	PTS mannose/fructose/sorbose transporter family subunit IID
LGAS_RS01085	-	4.43	sugar ABC transporter permease
LGAS_RS00905	-	2.93	6-phospho-beta-glucosidase
LGAS_RS00565	-	1.97	N-acetylglucosamine-6-phosphate deacetylase
LGAS_RS08305	-	2.64	PTS transporter subunit EIIC
LGAS_RS02470	-	2.46	6-phospho-beta-galactosidase
LGAS_RS00585	-	2.23	PTS fructose transporter subunit IIA
LGAS_RS07020	-	2.87	PTS sugar transporter subunit IIC
LGAS_RS01075	-	4.01	extracellular solute-binding protein
LGAS_RS01080	-	4.34	sugar ABC transporter permease
LGAS_RS00575	-	2.24	PTS fructose transporter subunit IIC
LGAS_RS08905	-	1.58	PTS sugar transporter subunit IIC
LGAS_RS00930	-	2.90	family 1 glycosylhydrolase
LGAS_RS01690	-	2.19	6-phospho-beta-galactosidase

Table III.3. *C. glabrata* DEG belonging to the COG carbohydrate metabolism and transport. Table describes the gene ID, the log2 foldchange (Log2FC) after 2h and 8h, the *S. cerevisiae* ortholog and a brief description of the gene (Part 1).

Gene ID	2 hours Log2FC	8 hours Log2FC	*S. cerevisiae* ortholog	Description
HXK2	-	4.09	*HXK2*	Putative hexokinase B
HXT1	-	4.91	*HXT1*	Ortholog(s) have fructose transmembrane transporter activity, pentose transmembrane transporter activity, role in glucose transport, mannose transport and plasma membrane localization
MIG1	-	2	*MIG1*	Transcriptional regulatory protein
CAGL0M08756g	-10.43	2.05	*EXG2*	Putative exo-1,3-beta-glucanase; predicted GPI-anchor
CAGL0H02387g	-1.45	-2.96	*TPS3*	Putative trehalose-6-phosphate synthase/phosphatase; gene is up-regulated in azole-resistant strain
CAGL0M03377g	-12.23	-3.76	*GLC3*	Ortholog(s) have 1,4-alpha-glucan branching enzyme activity, role in glycogen biosynthetic process
CAGL0H04939g	-1.20	-3.87	*FBP1*	Ortholog(s) have fructose 1,6-bisphosphate 1-phosphatase activity, role in gluconeogenesis, reactive oxygen species metabolic process and cytosol, periplasmic space localization
HXT5	-1.89	-2.71	*HXT3*	Ortholog(s) have glucose transmembrane transporter activity, role in glucose transport and plasma membrane localization
HXT4/6/7	-1.41	-4.40	*HXT6*	Ortholog(s) have glucose transmembrane transporter activity and mitochondrion, plasma membrane localization
GLK1	-1.40	-2.86	*EMI2*	Aldohexose specific glucokinase
PYK1	-17.66	-	*CDC19*	Pyruvate kinase
MDE1	-8.83	-	*MDE1*	Ortholog(s) have methylthioribulose 1-phosphate dehydratase activity, role in L-methionine salvage from methylthioadenosine and cytosol, nucleus localization
CAGL0F04895g	-1.64	-	*GPH1*	Ortholog(s) have glycogen phosphorylase activity, role in glycogen catabolic process, response to heat and cell surface, cytoplasm, hyphal cell wall localization
CAGL0F08261g	-1.20	-	*ENO1*	Ortholog(s) have high molecular weight kininogen binding, phosphopyruvate hydratase activity
CAGL0C01397g	-1.37	-	*PFK26*	Ortholog(s) have 6-phosphofructo-2-kinase activity and role in fructose 2,6-bisphosphate metabolism
CAGL0M02981g	-10.69	-	*PRM15*	Ortholog(s) have phosphopentomutase activity, role in guanosine catabolic process, inosine catabolic process, purine ribonucleoside salvage and cytosol, nucleus localization
FBP26	-	-2.60	*FBP26*	Ortholog(s) have fructose-2,6-bisphosphate 2-phosphatase activity, role in glucose metabolic process and cytoplasm localization
GDB1	-	-2.87	*GDB1*	Ortholog(s) have 4-alpha-glucanotransferase activity, amylo-alpha-1,6-glucosidase activity, role in glycogen catabolic process and mitochondrion localization

Table III.3. *C. glabrata* down-regulated genes belonging to the COG carbohydrate metabolism and transport. Table describes the gene ID, the log2 foldchange (Log2FC) after 2h and 8h, the *S. cerevisiae* ortholog and a brief description of the gene (Part 2).

Gene ID	2 hours Log2FC	8 hours Log2FC	*S. cerevisiae* ortholog	Description
CAGL0I04048g	-	-3.17	*FBP1*	Ortholog(s) have fructose 1,6-bisphosphate 1-phosphatase activity, role in carbon utilization, cellular response to glucose starvation and cytosol, nucleus localization
CAGL0E05610g	-	-2.58	*PYK2*	Ortholog(s) have pyruvate kinase activity, role in canonical glycolysis, pyruvate biosynthetic process and cytosol, mitochondrion localization
CAGL0K05137g	-	-3.70	*ATH1*	Ortholog(s) have alpha,alpha-trehalase activity and role in cellular response to desiccation, cellular response to ethanol, cellular response to freezing, pathogenesis, trehalose catabolic process
CAGL0G02717g	-	-2.91	*SGA1*	Ortholog(s) have glucan 1,4-alpha-glucosidase activity, role in glycogen catabolic process and Golgi apparatus, endoplasmic reticulum, fungal-type vacuole, prospore membrane localization
AQY1	-	-2.63	*AQY1*	Has domain(s) with predicted transporter activity, role in transport and membrane localization
PGM1	-	-2.86	*PGM1*	Putative phosphoglucomutase
TPS2	-	-3.06	*TPS2*	Ortholog(s) have trehalose-phosphatase activity

Amino acid metabolism and transport

The COG class related to amino acid metabolism and transport was another functional category comprising a high number of the *L. gasseri* genes found to be up-regulated along co-cultivation with *C. glabrata* (Figure III.6). This effect was more prominent at 8h than at 2h which can be attributable to a higher exhaustion of amino acids from the growth medium at this later time-point. A more detailed analysis of the genes clustered in this functional analysis allows us to see that it includes the amino acid permeases *LGAS_RS02795*, *LGAS_RS08890*, *LGAS_RS04265*, *LGAS_RS08770* and *LGAS_RS07680*; the oligoendopeptidase F (*LGAS_RS08435*), two branched-chain amino acid transporters *LGAS_RS00240* and *LGAS_RS04660*; and an N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase (*LGAS_RS07030*), among others (Table III.4).

While in *L. gasseri* the genes clustered in this COG class were exclusively up-regulated, the *C. glabrata* genes clustered in this class showed differential behaviour with some being up-regulated (e.g., *CAGL0F02233g*, *ARG8* and *ARG1*, involved in arginine biosynthesis) while others were down-regulated (e.g., *MET6*, *MET13* and *CAGL0L12254g*, involved in methionine biosynthesis) (Table III.5). Finally, was identified a set of genes to be down-regulated at both time points, including *CAGL0M00550g*, *CAGL0M04499g*, *CAR2* and *CAGL0E05632g* (Table III.5).

Table III.4. *L. gasseri* up-regulated genes assigned to the COG amino acid metabolism and transport. Table describes the gene ID, the log2 foldchange (Log2FC) after 2h and 8h and a brief description of the gene.

Gene ID	2h Log2FC	8h Log2FC	Description
LGAS_RS08195	1.10	-	oligopeptide ABC transporter substrate-binding protein
LGAS_RS02795	-	1.90	amino acid permease
LGAS_RS08435	-	2.61	oligoendopeptidase F
LGAS_RS08890	-	1.51	amino acid permease
LGAS_RS04265	-	1.72	amino acid permease
LGAS_RS00225	-	1.44	amino acid permease
LGAS_RS08770	-	1.96	amino acid permease
LGAS_RS00240	-	2.03	branched-chain amino acid transporter II carrier protein
LGAS_RS07680	-	1.82	amino acid permease
LGAS_RS04660	-	2.94	branched-chain amino acid transport system II carrier protein
LGAS_RS01280	-	1.43	APC family permease
LGAS_RS07030	-	1.90	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase

Table III.5. *C. glabrata* differentially expressed genes assigned to the COG amino acid metabolism and transport. Table describes the gene ID, the log2 foldchange (Log2FC) after 2h and 8h, the *S. cerevisiae* ortholog and a brief description of the gene (Part 1).

Gene ID	2h Log2FC	8h Log2FC	*S. cerevisiae* ortholog	Description
CAGL0M00550g	-8.79	-2.55	*STR2*	Ortholog(s) have cystathionine gamma-synthase activity, role in transsulfuration and cytosol, nucleus localization
CAGL0M04499g	-9.36	-2.52	*PUT1*	Ortholog(s) have proline dehydrogenase activity, role in proline catabolic process to glutamate and mitochondrion localization
CAR2	-10.14	-3.02	*CAR2*	Putative ornithine aminotransferase; protein abundance increased in ace2 mutant cells
CAGL0E05632g	-1.18	-3.67	*PUT4*	Ortholog(s) have L-proline transmembrane transporter activity, role in gamma-aminobutyric acid transport, proline transport and Golgi apparatus, endoplasmic reticulum localization
STR3	1.98	-2.68	*STR3*	Putative cystathionine beta-lyase; gene is up-regulated in azole-resistant strain
GLN1	1.85	-	*GLN1*	Ortholog(s) have glutamate-ammonia ligase activity, role in ammonia assimilation cycle, glutamine biosynthetic process and cytosol, nuclear periphery localization
CAGL0F02233g	-	2.20	*YFR006W*	Ortholog(s) have dipeptidase activity and cytoplasm localization
ARG8	-	4.31	*ARG8*	Ortholog(s) have N2-acetyl-L-ornithine:2-oxoglutarate 5-aminotransferase activity, role in arginine biosynthetic process via ornithine, ornithine biosynthetic process and mitochondrial matrix, nucleus localization
CAGL0K12232g	-	2.34	*ARO7*	Ortholog(s) have chorismate mutase activity, role in aromatic amino acid family biosynthetic process, sporocarp development involved in sexual reproduction and cytosol, nucleus localization
CAGL0F00253g	-	2.17	*TRP3*	Ortholog(s) have anthranilate synthase activity, indole-3-glycerol-phosphate lyase activity, phosphoribosylanthranilate isomerase activity, role in tryptophan biosynthetic process and cytosol localization
ARG1	-	2.15	*ARG1*	Argininosuccinate synthetase
CAGL0M06017g	-10.15	-	*TYR1*	Ortholog(s) have prephenate dehydrogenase (NAD+) activity, role in tyrosine biosynthetic process and cytosol localization
SPE4	-10.80	-	*SPE4*	Ortholog(s) have spermine synthase activity, role in pantothenate biosynthetic process, spermine biosynthetic process and cytoplasm localization
LYS9	-1.07	-	*LYS9*	Putative saccharopine dehydrogenase
CAGL0M11484g	-14.26	-	*ARO1*	Ortholog(s) have cytosol localization
HOM6	-12.20	-	*HOM6*	Ortholog(s) have homoserine dehydrogenase activity, role in homoserine biosynthetic process, methionine biosynthetic process, threonine biosynthetic process and cytoplasm, nucleus localization

Table III.5. *C. glabrata* differentially expressed genes assigned to the COG amino acid metabolism and transport. Table describes the gene ID, the log2 foldchange (Log2FC) after 2h and 8h, the *S. cerevisiae* ortholog and a brief description of the gene (Part 2).

Gene ID	2h Log2FC	8h Log2FC	*S. cerevisiae* ortholog	Description
CAGL0M09636g	-8.14	-	*TPP1*	Ortholog(s) have double-stranded DNA binding, polynucleotide 3'-phosphatase activity and role in double-strand break repair
CAGL0M04059g	-9.28	-	*PHA2*	Ortholog(s) have prephenate dehydratase activity, role in L-phenylalanine biosynthetic process and cytosol, nucleus localization
CAGL0M12188g	-10.09	-	*GCV3*	Ortholog(s) have glycine dehydrogenase (decarboxylating) activity, role in glycine catabolic process, one-carbon metabolic process, protein lipoylation and mitochondrion localization
CAGL0M08272g	-10.74	-	*TPO5*	Ortholog(s) have polyamine transmembrane transporter activity, role in cellular response to drug, polyamine transport and Golgi apparatus, endoplasmic reticulum localization
GAP1	-	-3.63	*GAP1*	Ortholog(s) have L-proline transmembrane transporter activity, polyamine transmembrane transporter activity and role in amino acid transmembrane transport, nitrogen utilization, polyamine transport
CAGL0D04026g	-	-2.42	*UGA1*	Ortholog(s) have 4-aminobutyrate transaminase activity, pyridoxal phosphate binding activity and role in cellular amide catabolic process, gamma-aminobutyric acid catabolic process, glutamate metabolic process
PRO1	-	-2.77	*YHR033W*	Putative gamma-glutamyl phosphate reductase
CAGL0K12518g	-	-2.79	*AGX1*	Ortholog(s) have alanine-glyoxylate transaminase activity, role in glycine biosynthetic process, by transamination of glyoxylate and biofilm matrix, cytosol, mitochondrion localization
MET6	-	-2.89	*MET6*	5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase; protein abundance increased in ace2 mutant cells
MET13	-	-2.66	*MET13*	Ortholog(s) have methylenetetrahydrofolate reductase (NAD(P)H) activity, role in methionine biosynthetic process, one-carbon metabolic process and mitochondrion localization
CAGL0G06732g	-	-2.58	*LEU9*	Ortholog(s) have 2-isopropylmalate synthase activity, role in leucine biosynthetic process and mitochondrion localization
CAGL0L12254g	-	-2.71	*ALT1*	Ortholog(s) have L-alanine:2-oxoglutarate aminotransferase activity, role in alanine biosynthetic process, alanine catabolic process, cellular response to drug, chronological cell aging and cytosol, mitochondrion, nucleus localization

Translation and ribosome biogenesis

Many of the *C. glabrata* differentially expressed genes while co-cultivated with *L. gasseri* were involved in translation and ribosome biogenesis, something that was not observed with their bacterial counter-partners genes whose expression was not found to be affected by the presence of the yeast. It was also noticeable the differential pattern of expression observed for these genes being mostly down-regulated at the 2h of co-cultivation, but up-regulated at 8h (Figure III.6). Remarkably, there was a very small overlap (of 2 genes only, *CAGL0M10197g*, encoding a ribosomal large subunit biogenesis and *CAGL0M06941g*, encoding a rRNA (cytosine-C5-)-methyltransferase) between these two datasets indicating that the genes that are down-regulated at 2h do not coincide with those that are later on up-regulated. In particular, after 2 hours of co-cultivation, a down-regulation of 15 ribosomal-encoding proteins genes was detected (e.g., *CAGL0M12408g*, *CAGL0M06303g*, *CAGL0M02497g* and *CAGL0M10241g*), as well as of the translation initiation factor *CAGL0M12144g*, and the threonine-tRNA ligase *CAGL0M12991g*, among others (Figure III.6 and Annex Table III.6). In contrast, after 8 hours of co-cultivation with *L. gasseri* 17 ribosomal-encoding protein genes were up-regulated in co-cultivation after 8 hours (e.g., *CAGL0A01562g*, *CAGL0E04994g*, *CAGL0D00616g*, *CAGL0G01078g*). Strikingly contrasting with what was observed for *C. glabrata*, co-cultivation only led to down-regulation of two *L. gasseri* ribosomal-encoding proteins, *LGAS_RS02200* and *LGAS_RS00820*, which can be due to an already smaller growth of the bacterial population (that was already inoculated at a higher density and therefore had less room to grow) both in single culture and in co-culture or it can result from protein synthesis being less susceptible to growth variations in this species.

Transcription

A considerable number of *C. glabrata* and *L. gasseri* DEGs in the co-culture were clustered in transcription functional class (11 and 7, respectively, after 2h; 13 and 5, respectively, after 8h). In the case of *C. glabrata* transcription-related genes were up-regulated after 8 hours but down-regulated at the early time point (Figure III.6). In specific, we found that the *C. glabrata* genes up-regulated included the RNA polymerases genes *CAGL0J01848g*, *CAGL0E05500g*, *CAGL0L03872g*, *CAGL0L02799g*, *CAGL0I07799g*, *CAGL0D06160g*, *CAGL0E02453g*, *CAGL0F00561g* and *CAGL0B04125g,* two genes encoding proteins involved in mRNA splicing (*CAGL0A03051g* and *CAGL0C02497g*) and a gene encoding a protein with ATP-dependent DNA helicase activity (*CAGL0F05643g*). On the other hand, the *C. glabrata* genes clustered in this functional class down-regulated at 2h of co-cultivation included the transcription initiation factor *CAGL0M05335g*; the transcription factors *CAGL0M00594g*, *ACE2*, *CRZ1* and *MSN4* (Figure III.6 and Annex Table III.4). This differential pattern of gene expression likely reflects the need of *C. glabrata* to adjust its genomic expression along the period of co-cultivation with *L. gasseri* that will likely involve different players. In *L. gasseri* we could also observe a differential pattern of expression of these genes that have transcription-related functions with some being up-regulated at 2h (such as the MarR regulator *LGAS_RS07000* or repressor of trehalose operon

LGAS_RS0263), while others were up-regulated at 8h (such as the heat-inducible transcriptional repressor HrcA and the GntR transcriptional regulator *LGAS_RS00935*) (Figure III.6 and Annex Table III.4).

Defense

In *C. glabrata* the genes included in the "defense response" COG class included the multidrug resistance transporters *CDR1* and *PDH1,* both down-regulated at 2h and 8h, respectively (Annex Table III.4). It also included the MFS transporters *FLR1* and *CAGL0B02343g*, although these were up-regulated after 8h of growth of the yeasts in the presence of the bacterium (Annex Table III.4). The down-regulation of these multidrug resistance transporters from *C. glabrata* along co-cultivation with *L. gasseri* is interesting in light of the demonstration that the efficacy of azoles against *Candida*, to whom these transporters confer resistance (as reviewed in Sarah G. Whaley and P. David Rogers, 2016[620]), is significantly enhanced in the presence of probiotic lactobacilli species, including in the presence of *L. gasseri*[369, 373]. This functional class also included several genes involved in response to oxidative stress in *C. glabrata* and that were up-regulated such as *TSA1* (thioredoxin peroxidase), *TRR2* (thioredoxin reductase) and *CTA1* (catalase A) (Annex Table III.4). Several *L. gasseri* genes clustered in this functional class were also found to be differentially expressed such as the ABC transporters *LGAS_RS05100*, *LGAS_RS06935* and *LGAS_RS05105,* up-regulated after 8h of co-cultivation, or *LGAS_RS04480*, encoding a hemolysin III family protein. Interestingly, other proteins of this family were before reported to inhibit yeast-to-hyphae transition in *C. albicans*[621]. *LGAS_RS02335*, encoding helveticin J, the sole bacteriocin that we could found in the genome of *L. gasseri*, was also found to be down-regulated in the co-culture setting (by 2.5- and 4.5-fold, respectively at 2 and 8h of co-cultivation).

III.4.3. Identification of genes improving *C. glabrata* competitiveness in the presence of *L. gasseri*

The genes differentially expressed by *C. glabrata* in the presence of *L. gasseri* (and vice-versa) could, at least in part, modulate the competitiveness of the yeast while growing in the presence of the bacterium. In particular, the up-regulated genes can be hypothesized to improve growth of *C. glabrata* while in the presence of *L. gasseri*. In this context, we have compared growth of wild-type *C. glabrata* KUE100 cells and of a selected set of mutants devoid of 5 *C. glabrata* genes found to be up-regulated in the co-culture setting: Cg∆*CAGL0L03828g*, Cg∆*CAGL0F04499g*, Cg∆*CAGL0L10208g*, Cg∆*str3* and Cg∆*rhr2* (Annex Table III.4). *C. glabrata CAGL0L03828g* encodes a protein predicted to have electron transfer activity with a role in sterol biosynthesis; *CAGL0F04499g* is a predicted uridine transporter; and *CAGL0L10208g* encodes a phosphopantothenoylcysteine decarboxylase, with an anticipated role in CoA biosynthesis[622-624]. Only the mutants devoid of *CAGL0L03828g*, *CAGL0F04499g* and *CAGL0L10208g* genes exhibited increased susceptibility to *L. gasseri*, showing a decrease in viability when co-cultivated

with the bacteria of 65%, 61% and 56%, compared with a decrease of 37% in viability observed for cells of the parental strain. The deletion of *STR3* and *RHR2* led to only marginal decreases in susceptibility to *L. gasseri* (Figure III.7).

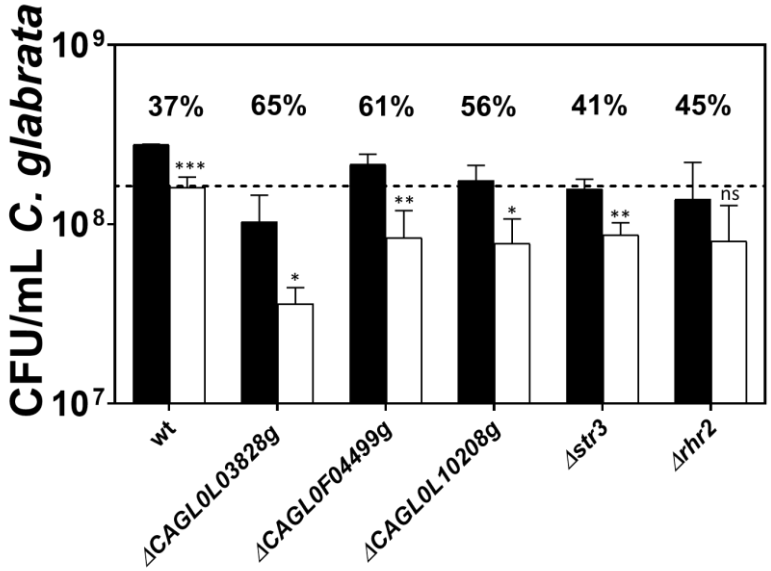


Figure III.7. Growth, in the presence and absence of *L. gasseri*, of single deletions mutants of *C. glabrata* devoid of genes that were up-regulated after 2 hours (Cg∆*CAGL0L03828g*, Cg∆*CAGL0F04499g*, Cg∆*CAGL0L10208g*, Cg∆*str3*) or 8 hours (Cg∆*rhr2*) in co-cultivation settings. Growth was performed for 96 hours at 37°C and 100 rpm. Cellular viability was determined based on the CFU method described in the material and methods section. The viability in co-cultivation (white bars) was compared with the viability in single cultivation (black bars) and the percentage of inhibition of the strains is given. Statistical significance was calculated using unpaired t-test (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001).

III.4.4. Identification of *L. gasseri* genes required for survival in the presence of *C. glabrata*

Similar to the above described exploration of the transcriptomics data to identify among the up-regulated genes those improving competitiveness of *C. glabrata* while in the presence of *L. gasseri*, we also tried to identify among *L. gasseri* up-regulated genes those improving competitiveness of this bacterium in the co-culture setting . To do this we chose to delete three genes from the genome of *L. gasseri*: *LGAS_RS04480*, encoding an hemolysin III; *LGAS_RS00970*, encoding a protease; and *LGAS_RS08435*, encoding an oligoendopeptidase F. The selection of these genes was based on their anticipated role in determining the interaction considering their biological function and also on their observed up-regulation in the co-culture with *C. glabrata*, by 3.4-, 3.7- and 6.1-fold, respectively. To undertake the genetic engineering of *L. gasseri* a collaboration with the laboratory of Prof Sarah Lebeer, at the University of Antwerp, was settled. In this context, I stayed at Lebeer's laboratory for approximately 3 months taking the first steps into the implementation of a strategy that would allow the envisaged gene deletions in this poorly characterized lactobacilli species. Such strategy, which is schematically represented in Figure III.8, is based on Gibson assembly and involves the construction of a vector that has a backbone, a tetracycline resistance marker in between two DNA fragments that comprise the flanking regions of the gene we want to eliminate. Transformation of *L. gasseri* cells with such

vector would allow the targeted gene deletion, after a double homologous recombination event, resulting in the replacement of the target gene by the tetracycline marker. However, during the course of my stay in Belgium and until the conclusion of this thesis it was not yet possible to the envisaged vector to be ready to be transformed into *L. gasseri*.

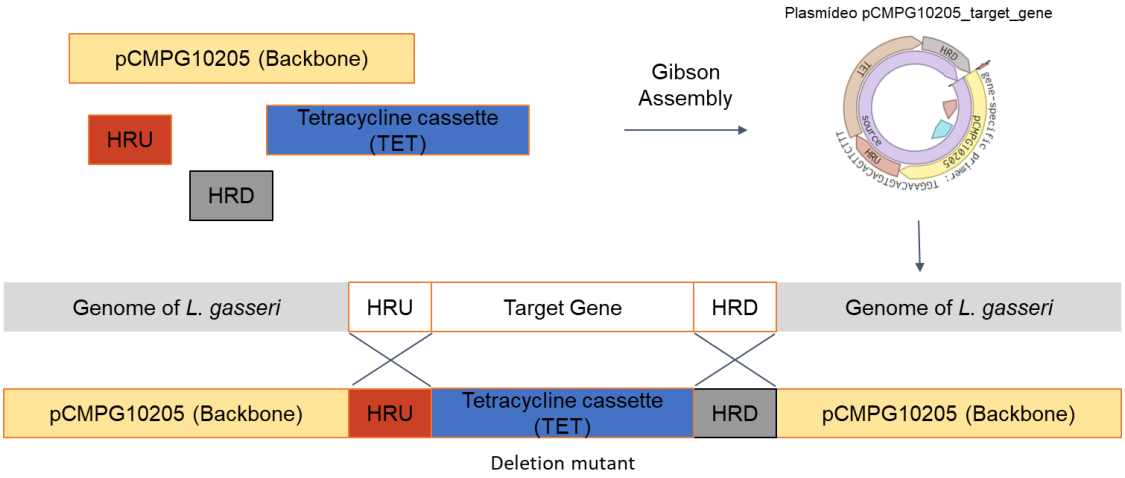


Figure III.8. Gibson assembly strategy used to construct the *L. gasseri* single deletion mutants. For this was used the plasmid pCMPG10205 with the resistance markers for erythromycin and tetracycline. First is amplified the upstream and downstream homologous regions of the target genes and the amplification of the tetracycline from the backbone plasmid. After Gibson assembly of these fragments, including the linear backbone, it would be inserted in the cells of *L. gasseri* and the target gene would be replaced by the tetracycline genes by double homologous recombination.

III.5. Discussion

Considering the harmful impact of vaginal candidiasis in women health, along with its economic impact, it is crucial the development of alternative anti-*Candida* therapies. In this context, research aiming to explore probiotic lactobacilli (especially those indigenous to human microbiomes) as eventual anti-*Candida* agents have been gaining traction and in this work we address this by focusing the interaction established between *C. glabrata* and the poorly studied vaginal species *L. gasseri*. After demonstrating in the previous chapter that such co-cultivation results in reduced viability of the yeast cells, a phenotype modulated by the presence of acetate in the medium, in this chapter we undertake a molecular analysis of these co-cultivated cells using a dual RNA-seq approach. Importantly, *L. gasseri* has already demonstrated good potential to serve as anti-*Candida* agents in clinical trials[373] and the understanding of the factors that can modulate this interaction may help guide more efficient approaches for its successful exploration as an anti-*Candida* platform. Besides shedding light into the interaction established between *L. gasseri* and *C. glabrata*, the herein undertaken transcriptomic analysis also advanced the annotation genome of the reference strain *L. gasseri* ATCC 33323 and at a lower extent the one of *C. glabrata* CBS138. In specific, detailed analysis of the read mapping obtained from the RNA-seq to the genome sequence of *L. gasseri* ATCC 33323 identified 30 transcripts likely to encode genes that are not annotated but should. We believe some of these are real genes because they encode proteins described in other species (in some cases even in other *L. gasseri* strains), although most of them with uncharacterized functions. Similarly, two genes not annotated in the genome of the *C. glabrata* CBS138 strain (Novel_870 and Novel_1656, encoding two uncharacterized proteins) were also uncovered based on their differential pattern of expression in the single- and in the co-cultures with *L. gasseri*. Besides this, a set of possible non-coding RNAs were also identified in this work, both in *L. gasseri* and in *C. glabrata* (listed in Annex Table III.3). In most cases, these novel RNA transcripts mapped in the opposite strand of annotated genes, while in a few other cases these were mapped in intergenic regions. While the first can be considered as possible cis-sRNAs and likely affect the expression of the gene where they are located at by preventing transcription due to the formation of an RNA duplex with the mRNA they encode; the second ones are trans-sRNAs and therefore will have a more pleiotropic effect much harder to dissect with the data available. In this context, it is interesting that the up-regulation of the cis-sRNA identified as CgNovel_981 (mapped to region 553922 to 555499 in chromosome E) was accompanied by a concomitant down-regulation of the *CAGL0E05610g* gene. Similarly, up-regulation of LgNovel_8 (see Annex Table III.3) and LgNovel_261 (see Annex Table III.3) was accompanied by a down-regulation of the gene located where they mapped, *LGAS_RS00380* and *LGAS_RS09035*. However, from the remaining predicted sRNAs (detailed in Annex Table III.3) most had a pattern of expression that was identical to the one of the gene they originate from. We cannot rule out that these predicted antisense RNAs can also be artifacts originated during the preparation of the library used for the sequencing, however, the fact that they are detected in all the samples that had been used for the sequencing reduces this probability as in

the case of artifacts we would expect to obtain a random distribution of appearance (they would be produced in some samples but in not in others and therefore they would be discarded along the RNA sequencing analysis pipeline that requires information obtained from all the samples). It is also relevant to say that among the 1777 predicted anti-sense transcripts hypothesized in *C. glabrata*, 157 were previously recognized as possible non-coding RNAs in this species (Annex Table III.3)[616].

A substantial portion of the genes from *C. glabrata* and *L. gasseri* could not be attributed to a specific COG functional annotation, which can indicate that this interaction might involve a relatively set of genes that could be specifically involved in this type of interactions and since these are relatively under-explored they remain to be uncharacterized (this especially true in the case of the *C. glabrata* differently expressed genes); but also the lack of knowledge about physiology and biology of *L. gasseri*. Notably, the herein reported transcriptomic changes in *C. glabrata* while growing the presence of *L. gasseri* show similarities to those observed in *C. albicans* while growing in the presence of *L. rhamnosus* and *L. crispatus*[140, 398]. Specifically the presence of *L. rhamnosus* and *L. crispatus* induced changes in genes related with amino acid and carbohydrate transport and metabolism[140, 398] reflecting competition of the species for these crucial nutrient sources, although it is important to denote important differences in the experimental settings used in the different experiments (the interaction with *L. crispatus* was examined in a biofilm and the interaction with *L. rhamnosus* in a tri-culture setting that also involved epithelial intestinal cells). In particular, it was found a down-regulation of *C. glabrata* genes involved in carbon metabolism, specifically genes with a possible role in glycolysis, similar to what was previously observed in the response of *C. albicans* to the presence of *L. rhamnosus*[398]. Additionally, it was observed in both yeast species while growing in the presence of the different lactobacilli species an up-regulation of two enzymes involved in oxidative phosphorylation, succinate dehydrogenase (*CAGL0C03223g*) and cytochrome c (*CYC1*). Presumably an impaired oxidative phosphorylation could limit ATP production, which could reduce fungal growth, filamentation, and virulence as previously demonstrated[606]. From our data set was also observed an up-regulation of two genes involved in lactate consumption (*CYB2* and *DLD1*). Although their up-regulation was not possible to be correlated with a consumption of lactic acid in these early timepoints we have observed a decrease of the lactic acid concentrations from 5 g/L at 24h of growth to 3.5 g/L at 96h of growth which indicates a consumption of this weak acid by *C. glabrata* (Annex Figure II.4 panel A). In *C. albicans* no correlation between the presence of lactobacilli and the up-regulation of genes responsible for lactate utilization was described. While we could not detect a differentially expression of *L. gasseri* genes responsible for production of lactic acid we have observed that in co-cultivation its concentration is lower than the one observed in *L. gasseri* single cultures (Annex Figure II.4 panel A.). However, we could not disclose if this decrease was a consequence of a lower ability of *L. gasseri* to produce lactic acid, the ability of *C. glabrata* to consume it or even a consequence of both.

An interesting observation was the observed prominent up-regulation of *C. glabrata* genes involved in translational, ribosomal structure and biogenesis regulation during the co-

cultivation that was not observed to occur at a significant extent for *L. gasseri* and was also not observed in the case of *C. albicans*[140, 398]. It is possible that these differences found in the pattern of expression of these genes related with protein synthesis reflect the different state of the cells in co-culture and in single-culture (which was what we used as controls), being the cells in co-culture growing more actively (and thus requiring higher protein synthesis) at 8h, compared to the single-culture at the same time point that is already approaching the stationary phase; while at 2h it is the single-culture that is growing more actively since the co-inoculation with *L. gasseri* reduced the growth rate and resulted in some delay in growth.

Modified expression of the *C. glabrata* hexose transporters along co-cultivation with *L gasseri* was also observed with *HXT1* being up-regulated, while *HXT5* and *HXT4/6/7* were down-regulated. Studies show that like *S. cerevisiae*, *C. glabrata* adjusts the expression of HXT genes according with the glucose concentration in the environment, presumably as a mean to optimize consumption since these transporters have different affinities for glucose[625]. Previous reports have shown that *HXT1* and *HXT4* expression was identical in media with low or high glucose conditions[625] suggesting that they have constitutive expression; while, *HXT3* and *HXT5* are believed to be high affinity hexose transporters as their expression was higher when glucose was scarce in the environment[625]. In co-cultivation we have a quick consumption of glucose from the broth resulting in its complete utilization after 24h of growth. However, in the 2h and 8h timepoints we have not examined the glucose concentration of the broth, this being a task to be performed in the near future. Nonetheless, we could observe that *C. glabrata* had different HXT gene expressions, with an up-regulation of *C. glabrata HXT1* in co-cultivation, while *HXT5* and *HXT4/6/7* were down-regulated. With the information gathered so far it is not possible to correlate the pattern of expression of these HXT genes with a glucose starving state induced by *L. gasseri*, however it remains as an interesting topic to be explored in the future. In *L. gasseri* co-cultivation also prompted an up-regulation of the genes involved in phosphotransferase (PTS) sugar transport system (*LGAS_RS02465*, *LGAS_RS00730* and *LGAS_RS08210)* suggesting an attempt to improve the uptake of this sugar from the environment. Although PTS system has not yet been specifically studied for the *L. gasseri* species, in *L. acidophilus* the expression of these PTS genes was found to increase in response to reduced glucose concentration in the environment[626].

In the same line several genes related to amino acid transporters and metabolism were also differentially expressed in the co-cultivation, both in *C. glabrata* and in *L. gasseri*. In particular, it was observed an over-expression of *C. glabrata* genes involved in ammonia assimilation (*GLN1*) and in biosynthesis of arginine, tryptophan and of aromatic amino acids. In *L. gasseri* an up-regulation of a significant number of amino acid permeases was also observed (*LGAS_RS02795*, *LGAS_RS08890*, *LGAS_RS04265*, *LGAS_RS00225*, *LGAS_RS08770*, *LGAS_RS07680*, *LGAS_RS01280*) as well as of the two branched-chain amino acid transport system II carriers (*LGAS_RS00240*, *LGAS_RS04660*). Altogether, these observations suggest that both types of cells aim to evolve responses that might help them to improve amino acid uptake and subsequent biosynthesis reflecting what is an expected reduced availability of nitrogen sources in the co-

culture setting. Interestingly, in Chapter II we could observe that along co-cultivation with *L. gasseri*, *C. glabrata* (and also *C. albicans*) cells prompt an alkalinization of the growth medium, something that was also observed to occur in single-cultures even at a higher rate than the observed in the co-culture. Although we could not determine the nature of the alkalinizing agent, in *C. albicans* this was attributed to the excretion of amino acids and linked to the activity of the Stp2 transcription factor, regulator of amino acid permeases expression; of the acetyl-coenzyme A hydrolase Ach1; of the urea amidolyase *DUR1,2*; and of *ATO5*, a putative ammonia transporter[567, 568]. Notably, the expression of *CgDUR1,2* was reduced in the co-cultivation with *L. gasseri*, compared with the expression levels that these genes attained during single-cultivation of *C. glabrata* cells, with this pattern of expression being in line with the lower kinetics of alkalinization observed in the co-culture.

The co-cultivation with *L. gasseri* also induced the expression of genes linked with iron homeostasis in *C. glabrata* including *GRX4* and *CTH2*, encoding a putative glutathione-dependent oxidoreductase induced by excess iron and a protein involved in cellular iron homeostasis, respectively. These two genes are targets of the transcription factor Aft2, a key player in control of iron starvation response in *C. glabrata*[627] and although expression of *ATF2* transcription factor itself was not differentially expressed in the co-culture, we could find several other Atf2 documented targets also up-regulated in the co-cultivation setting, including *ROX1*, *CAGL0K07007g*, *CAGL0K08734g*, *CTA1*, *CAGL0G06248g, CAGL0B02343g* and *SAM1*. This observation suggests that it is possible that Aft2 is activated under the co-culture setting, a hypothesis that needs to be addressed with further detail. Lactobacilli species don't have specific iron requirements and this is believed to give them an advantage over other microbes in environments with scarce levels of iron, as it is the human infection sites[628, 629]. Despite this, it was interesting to observe in the co-culture an up-regulation of the *L. gasseri* hemolysin *LGAS_RS04480*, an enzyme that causes lysis of red blood cells resulting in the release of iron, that in other bacteria (*Vibrio vulnificus*, *Aeromonas hydrophila* and *S. aureus*) has also been found to be up-regulated in conditions of iron starvation[630-632]. This aspect is considerably important since the environment conditions of the host have a lower concentration of iron, thus competition for this nutrient can be crucial to guarantee the successful colonization of microorganisms. Therefore, would be interesting for the future to verify if co-cultivation between *L. gasseri* and *C. glabrata* results in the limitation of iron.

The production of hydrogen peroxide has been suggested to underlie the anti-*Candida* effect prompted by lactobacilli and therefore we searched our dataset of *L. gasseri* for differentially expressed genes encoding pyruvate oxidase, NADH oxidase, lactate oxidase, glycolate oxidase and NADH-dependent flavin reductases, shown to be the main sources of H_2O_2 production in *L. johnsonii*[633, 634]. We could not detect significant up-regulation of *L. gasseri* genes with the above-mentioned functions in the co-culture setting, however, several *C. glabrata* genes involved in anti-oxidative stress response (such as the thioredoxin peroxidase Tsa1, the thioredoxin reductase Trr2 and catalase Cta1) were up-regulated. This response prompted by the yeast cells suggests that indeed along the co-cultivation the cells appear to suffer from an increased oxidative

stress but it is not possible to know whether that is because the bacteria is indeed producing H_2O_2 which is afterwards affecting the yeasts, or if this merely reflect a higher metabolic activity of the yeasts that consequently results in enhanced production of ROS. It is however important to note that the lack of up-regulation of *L. gasseri* genes involved in production of H_2O_2 does not mean that these cells are not producing this metabolite at enhanced rates in the co-culture since it is possible that such production does not depend on higher levels of the enzymes whose activity may be increased by other means (e.g., higher activity due to post-translational modifications).

Previous works shown that *C. glabrata* Hog1, involved in response of yeasts to osmotic stress, is crucial for competitiveness against *Lactobacillus*, including *L. gasseri*[635]. Despite this, we could find the *HOG1* gene down-regulated in the co-culture at the early time point of adaptation, however, the past observation showing that Hog1p is required for competitiveness against lactobacilli were performed after 24h *C. glabrata* of cultivation. Thus, it is possible that these gene can be down-regulated at an early stage of growth but up-regulated in later stage. Additionally, it is important to mention that the activity of the Hog1 signalling pathway does not depend entirely of the genomic expression of *HOG1* gene because it is not regulated at a transcriptomic level, but regulated through protein phosphorylation[636, 637]. Lactic acid was showed to induce the translocation of Hog1p to the nucleus[635], thus lactic acid produced by lactobacilli could function as an activator of the HOG pathway. However, due to the complexity of a co-cultivation containing lactobacilli it could be possible that other environmental cues (e.g., other metabolites produced by lactobacilli) could also be responsible for the activation of Hog1p. Additionally, we could also observe a down-regulation of *CAGL0E03916g* (glycerol kinase) and *CAGL0H06699g* (glycerol-3-phosphate dehydrogenase) required for glycerol metabolism, resulting in its accumulation in the cytoplasm in co-cultivation conditions, possibly compensating an hyperosmolarity of the extracellular environment caused by *L. gasseri* produced metabolites that could also potentially activate the Hog1 pathway.

The production of bacteriocins is a common mechanism by which lactobacilli species antagonize other microbial species and some of these proteins have been found to have activity against *C. albicans* and *C. glabrata*[467, 468, 472]. The *L. gasseri* strains used in this work has been described to produce only helveticin, which was already reported to have antifungal properties against *C. glabrata* and *C. albicans*[218]. The gene encoding helveticin was found to be expressed by *L. gasseri* cells both when these were cultivated alone or in the presence of *C. glabrata*, with the expression being considerably lower in the co-culture setting. It remains to be established whether the levels of helveticin produced by the *L. gasseri* cells, even if lower in the co-culture, are sufficient to cause damages to *C. glabrata*. In this context it was also noticeable the higher expression of the hemolysin III family protein *LGAS_RS04480* in the co-culture since, like bacteriocins, these proteins were also shown to target the cell envelope structure, including the one of *C. albicans*[621].

C. glabrata genes found to be up-regulated by the presence of *L. gasseri* are candidates to be important in this interaction and assure survival of the yeast. Using that principle, we show

herein that the deletion of *CAGL0L03828g*, *CAGL0F04499g* and *CAGL0L10208g* genes, all up-regulated in the co-culture setting, increases susceptibility of *C. glabrata* to *L. gasseri*. *CAGL0F04499g* is an ortholog of the uridine permease *ScFUI1*[623], *CAGL0L10208g* is an orthologue of *ScVHS3*, involved in repression of signalling mediated by the Ppz1 phosphatase[638] and *CAGL0L03828g*, a predicted cytochrome b5 protein, is homologous to *ScCyb5* that in *S. cerevisiae* was shown to be involved in sterol and lipid biosynthesis pathways by compensating for the function of Erg11. While in the case of the uridine transporter it can be argued that the absence of this protein can reduce the ability of *C. glabrata* to uptake it from the medium, thus compromising the ability of the yeast to synthesize nucleic acids it is difficult to anticipate the reasons underlying why *C. glabrata* cells require the other two proteins for maximal survival in the presence of *L. gasseri* this requiring more studies that can specifically address this subject. In *C. albicans* the deletion of *CYB5* was found to affect the overall lipid composition of the plasma membrane (and because of that affecting tolerance to azoles) and therefore one can speculate that a similar thing can happen in this *C. glabrata* mutant, that may result in enhanced susceptibility to the products secreted by *L. gasseri* to the environment and, eventually, to the cell-cell contacts that may also occur, despite we are using agitation in the co-cultures. For future perspective would be important to evaluate if these genes are required for competitiveness of *C. glabrata* against lactobacilli vaginal isolates, which would reenforce the notion that these are potential new therapeutic targets to be used to treat vaginal candidiasis. Interestingly, several genes encoding *C. glabrata* adhesins were found to be up-regulated in the co-culture including *AWP3*, *AWP6* and *EPA1.* It is unclear why under these conditions such genes are up-regulated but it is possible that the high cellular density of the cultures used ($8.5x10^7$ CFUs/mL including both yeast and bacterial cells at 2h and $1.3x10^8$ at 8h) may somehow promote contacts between these cells whose outcome will be quite interesting to study. In the future it will also be important to investigate the set of up-regulated *C. glabrata* genes in a more systematic manner to search for others that might also contribute to maximize survival in the presence of *L. gasseri*. By helping the yeasts thrive in the presence of the bacteria, these genes can be targeted in assays designed to improve competitiveness of the microflora in detriment of *Candida*. In this context, it is particularly interesting that Fui1 was found to have high affinity to benzbromarone, an already approved drug for the treatment of gout and that has shown some capacity to inhibit *C. albicans*[639]. It will be interesting to test how the *C. glabrata*/*L. gasseri* equilibrium is affected in the presence of this pharmaceutical. It is exactly in this context of providing new therapeutic target that the search for competitiveness genes provides essential information. In Figure III.9 we tried to compile the different aspects of the *C. glabrata* – *L. gasseri* interaction and also on the herein undertaken analysis support, or contradict, the involvement of anticipated players in determining lactobacilli antimicrobial potential.

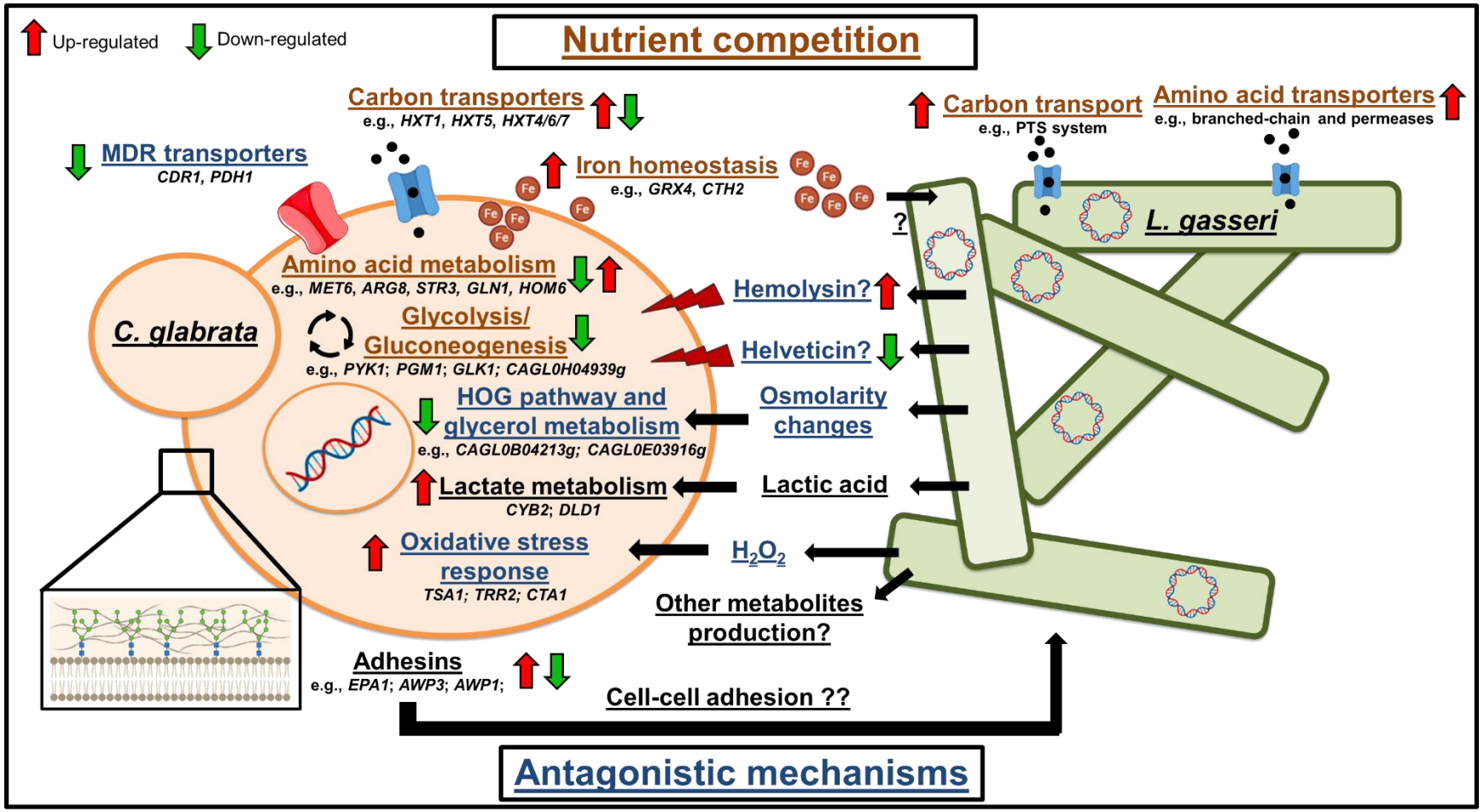


Figure III.9. Proposed model of adaptation of *C. glabrata* and *L. gasseri* interaction based on the RNA sequencing data. Co-cultivation induced changes in several genes related to carbon and amino acid transport and metabolism, MDR transporters, iron homeostasis and adhesins. Additionally, *C. glabrata* modulated the genomic expression of genes related to the oxidative and osmotic stress response. Production of helveticin, hemolysin and other metabolites, while still unclear, could be correlated with the anti-*Candida* potential of *L. gasseri*. Red arrows indicate the up-regulation while the green arrows indicate the down-regulation of genes associated with each biological function. Note that some biological functions are either up or down-regulated simultaneously. The list of DEGs used to construct this model is described in Annex Table III.4.

IV. A phenOMICs approach to identify genes determining *C. glabrata* survival along planktonic and biofilm co-cultivation with *L. gasseri*

IV.1. Abstract

Identification of genes determining the ability of *C. glabrata* genes contributing for capacity of these yeasts to thrive in the presence of *L. gasseri* provides an interesting cohort of novel targets that could be used in the development of new anti-*Candida* drugs, eventually based on probiotics. In this sense, in this work we profiled a collection of single deletion *C. glabrata* mutants for their ability to grow, in planktonic and in biofilm forming conditions, in the presence of *L. gasseri*. For the phenotypic screening undertaken in planktonic conditions a competitive pooled growth assay was performed leveraging the sequencing of barcodes that unequivocally identify each *C. glabrata* mutant strain. The screening performed in mixed biofilms formed between *C. glabrata* mutant strains and *L. gasseri* was performed resorting to a herein described experimental assay that involved a first screening using propidium iodide, complemented with confocal microscopic imaging of the biofilms, to search for yeast strains with reduced viability. Due to a number of technical problems, it was not possible to conclude the screening performed under the planktonic conditions, although it had been possible to establish the important role in determining survival of *C. glabrata* of genes involved in resistance to acidic environments (C*AGL0G08844g*), in osmotic sensing in the HOG signalling pathway (*CAGL0F08833g*), in ergosterol biosynthesis (*ERG5*) and the adhesin Epa1. Differently, the screening performed in biofilm-forming conditions was successfully completed and allowed us to conclude on the involvement of a cohort of genes that include *BCY1* from the PKA signalling pathway, *SHO1*, *HKR1*, *PBS2,* and *SMP1* from the HOG signalling pathway and *MID1*, *CCH1 CNA1* and *CRZ1* from the calcineurin signalling pathway. Despite the evidence of a possible role of these pathways in the survival of *C. glabrata* in mixed cultures, it was not identified the downstream effectors, possibly due to the redundancy of their role, or simply because they were not present in the mutant collection. In conclusion, this work was successful in developing two phenotypic screenings, that could be used in other contexts, and more importantly, identified possible mechanisms of competitiveness of *C. glabrata* that will be scrutinized in the future.

IV.2. Introduction

Despite the doubts remaining the extent at which vaginal lactobacilli species restrain the progress of vaginal candidiasis *in vivo*, the results obtained from *in vitro* studies and clinical strains sustain that some of these bacterial species do have a strong anti-*Candida* potential that can be leveraged to develop new anti-*Candida* therapies. Consequently, the interest in studying how these species interact with each other is high aiming to create foundational knowledge that can also help designing strategies that could favour the commensal vaginal lactobacilli microbiota, in detriment of *Candida*. The reduction (or even elimination) of the activity of genes and pathways that mediate the ability of *Candida* cells to thrive in the presence of vaginal lactobacilli species can be such a strategy. However, to date, this topic is still quite elusive. In a pioneer study, Reinhard Beyer *et al.*, described the involvement of the *C. glabrata* Hog1 protein as mediating the capability of this yeast to grow while in the presence of *L. crispatus*, *L. gasseri* and *L. rhamnosus*[635]. In *C. glabrata*, as in S. cerevisiae, the HOG (high osmolarity glycerol) pathway responds to variations in osmotic pressure[637]. In specific, under osmotic stress, the MAPK Hog1 protein is phosphorylated by MAPKK kinase Pbs2, which is itself phosphorylated by the MAPKKK kinases Ssk2, Ssk22 or Ste11 (Figure IV.1)[637, 640].

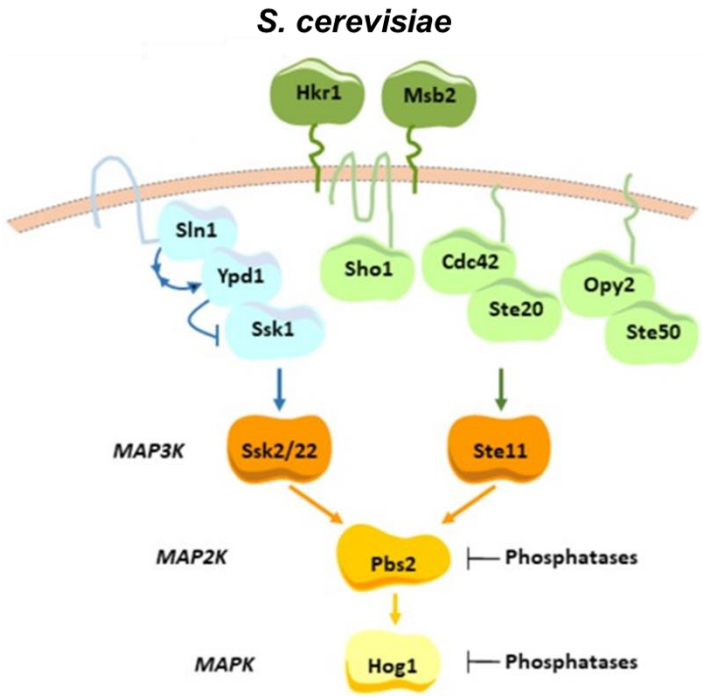


Figure IV.1. HOG signalling pathway architecture in *S. cerevisiae*. The two signalling branches (*SHO1* and *SLN1*) converge to phosphorylate the protein Pbs2 which directly regulates the Hog1 protein. Figure taken from Eulàlia de Nadal and Francesc Posas, 2022[637].

Although the role of the HOG signalling cascade in *C. glabrata* has been more studied in the context of environmental insults that are known to alter osmotic pressure (such as presence of high concentrations of sodium chloride, that cause hyperosmotic stress), the CgHog1 protein has also been demonstrated to be activated in response to stress imposed by weak organic acids[641, 642] or by high external iron levels in the environment[643]. Such incapacity to respond

to iron excess conditions was suggested to be linked with defects in iron metabolism related genes in the Cg*Δhog1* mutant[643]. Consistent with this hypothesis, the transcript level of *CgYAP5*, encoding a high iron-responsive transcriptional activator; Cg*FTH1*, encoding a vacuolar iron permease; and *CgCTR2*, encoding a low-affinity vacuolar copper transporter, were lower in the Cg*Δhog1* mutant, compared to the levels attained in cells of the parental strain[643]. Hog1 protein was also reported to be required for maximal survival of *C. glabrata* inside macrophages and increased this yeast virulence in the murine model of disseminated candidiasis[643]. Altogether, these observations suggest other additional roles for CgHog1 beyond the response to alterations of external osmotic pressure.

In the specific context of the role played by *C. glabrata* Hog1 along co-cultivation with lactobacilli, it was shown that deletion of *HOG1* gene resulted in reduced growth in the presence of *L. crispatus*, *L. gasseri* and *L. rhamnosus* along co-cultivation in MRS agar plates[635]. Consistently, in cells of the parental strain it was shown an increase in CgHog1 activity upon exposure to culture supernatants obtained from *L. rhamnosus*, with the levels of phosphorylated Hog1 increasing very rapidly after exposure of the cells to the bacterial supernatant, although at lower levels than those caused by osmotic stress, possibly because of the presence of lactic acid[635]. The adjustment of supernatant pH to neutral pH did not change the activation kinetics of the Hog1 protein[635]. Although the authors showed that lactic acid triggered Hog1 activation and a strain lacking this gene was susceptible to lactic acid under vaginal physiological conditions, they failed to elucidate if other compounds in the medium could also activate the HOG pathway leaving only open the hypothesis of the involvement of this signalling pathway in resistance to other individual stresses that could be caused by the presence of the bacteria such as oxidative, nitrosative, and other weak acid stress[635].

In the last chapter of this thesis, we have demonstrated the prominent role of *CAGL0L03828g*, *CAGL0F04499g* and *CAGL0L10208g* genes in maximizing growth of *C. glabrata* while in the presence of *L. gasseri*. The selection of these genes was based on their up-regulation during co-cultivation of *C. glabrata* with *L. gasseri*, however, the dataset of up-regulated genes was very large and not all genes required for competitiveness will have to be transcriptionally regulated. Thus, in this chapter we endeavoured on a large scale phenotypic screening using a commercial collection of *C. glabrata* mutant strains that had been before used to elucidate important aspects of the physiology of this species including antifungal tolerance mechanisms[644]. Besides searching for genes that could determine growth of *C. glabrata* in the presence of *L. gasseri* in planktonic conditions, we have also examined the viability of the different yeast strains while in a mixed biofilm with the bacterium considering that this is the expected way by which these cells are expected to stay *in vivo*.

IV.3. Material and Methods

IV.3.1. Strains and growth media

In this chapter it was used the bacterial strain *L. gasseri* ATCC33323 (acquired from DSMZ), *C. glabrata* ATCC2001 and the *C. glabrata* mutant collection (comprised of 578 single mutants)[644]. This collection is divided into two groups, built in different genetic backgrounds. Mutants belonging to group 1 were constructed in the background of *C. glabrata* ATCC2001 (auxotrophic for histidine, tryptophane and leucine; HTL) and include 438 strains. To build the mutant strains, the dominant recyclable nourseothricin resistance marker *SAT1* was used to replace the coding sequences of *HIS3*, *LEU2* and *TRP1* resulting in a triple auxotroph for histidine, leucine and tryptophan. The repeated use of this marker cassette resulted in the new *C. glabrata* background recipient strain for deletions referred to as HTL, to which was further added two 20 bp barcodes in the *trp1* locus using a *NAT1* cassette (HTL: *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::*NAT1* (barcoded)). Afterwards, replacement of the different genes was performed using the same strategy based on homologous recombination that had been used to replace the *HIS3*, *LEU2* and *TRP1* genes using a *NAT1* cassette containing a specific barcode. Mutants belonging to group 2 were engineered in the background of the ATCC2001_Δ*his3* mutant (not barcoded), a derivative of the background HTL (and thus only auxotrophic for histidine). The list of all 578 *C. glabrata* deletion strains phenotyped is described in Annex Table IV.1 and the specific barcode for their identification is described in Annex Table IV.2.

The *L. gasseri* and the *C. glabrata* strains were cultivated in MRS medium that contains, per liter, 10 g casein peptone (Gibco); 10 g meat extract (Panreac AppliChem); 5 g yeast extract (Gibco); 20 g glucose (Nzytech); 1 g Tween 80 (Sigma); 2 g K_2HPO_4 (Merck); 5 g sodium acetate (Merck); 3 g ammonium sulphate (Panreac AppliChem); 0.20 g $MgSO_4.7H_2O$ (Labchem) and 0.05 g $MnSO_4.H_2O$ (Sigma). After preparation, the pH of MRS was adjusted to 6.2-6.5 using HCl or NaOH. Solid YPD medium was used for maintenance of the yeast strains, and contained, per litre, 20 g glucose (Nzytech), 20 g peptone (Gibco) and 10 g yeast extract (Gibco) while the bacterial strain was kept at -80ºC in MRS and 86% glycerol. When required for plating solid YPD or MRS were prepared by supplementing the corresponding liquid medium with 2% and 1.5% agar (Nzytech), respectively. Media were prepared using deionized water and sterilized by autoclaving for 15 min at 121°C and 1 atm.

IV.3.2. Phenotyping of the *C. glabrata* mutant collection along planktonic co-cultivation with *L. gasseri*

IV.3.2.1. Mutant pooling

The *C. glabrata* mutant collection was rendered available in 7 96-multiwell plates, in each well being placed one deletion strain. For subsequent phenotyping during growth in co-cultivation

with *L. gasseri*, pools containing approximately 96 yeast strains were prepared. For that, strains were thawed and inoculated (using a 96-multiwell pin replicator) in a Petri dish (having 75mL of volume) containing solid YPD supplemented with 100 mg/L nourseothricin (resistance marker). The plates were incubated at 30ºC for 48h, after which 12mL of YPD supplemented with 100 mg/L nourseothricin were added to the surface of the agarised plates to resuspend the strains. In total, 7 pools were prepared for subsequent phenotyping. Besides the mutants, all pools also included the wild-type *C. glabrata* strain (ATCC2001 HTL or ATCC2001_Δ*his3,* depending on the cohort) that was added individually to each Petri dish. Slow-growing mutants were thawed individually and added to the pool of mutants. Pools were constructed taking into consideration the abundance of each mutant on the pool to be phenotyped. The cellular density of *C. glabrata* mutants during co-cultivation with *L. gasseri* was prepared to be around 10^6 CFU/mL in total, which corresponds to an average abundance of 10^4 CFU/mL for each mutant strain in 50 mL MRS medium. To standardize the inoculum between different pools (100 µL in 50mL of MRS medium), these were frozen with an optical density (OD_{600nm}) of 50, measured using a U-2001 Spectrophotometer from Hitachi, and adjusted to 15% glycerol before storage at -80ºC until further use.

IV.3.2.2. Phenotyping of the mutant collection

Each pool of ~96 *C. glabrata* mutants (in a total of 7) was thawed and 100 µL of it were diluted in 50 mL of fresh MRS (control) or in MRS already containing a population of *L. gasseri* having an OD_{600nm} of 0.4. With this setup, the cellular density of the *C. glabrata* mutant pool was around 10^6 CFU/mL in total that corresponds to an average abundance of 10^4 CFU/mL for each mutant strain. The pooled yeast cultures, either exposed to *L. gasseri* or not, were incubated in a shaking incubator at 37ºC and 100 rpm for 24h. After this time, 1 mL of growth culture was collected and centrifuged at 13500 rpm for 2 minutes and the cell pellet obtained was harvested and stored at -80ºC until further use. Total DNA was extracted from each cell pellet. For that, 200 µL of lysis buffer [50 mM Tris-HCL; 50 mM EDTA; 250 mM NaCl; and 0.3 % SDS] were added to each cell pellet and the suspension vortexed for 2 minutes at maximum speed, followed by an incubation at 65ºC for 1 hour. After this time, the cellular suspension was placed on ice for a few minutes and vortexed again for 2 minutes at maximum speed. The suspension was centrifuged for 15 minutes at 13000 rpm at 4ºC and the supernatant collected to a new Eppendorf tube. To recover the DNA, 20 µL of sodium acetate 3 M (at pH 4.8) and 400 µL cold ethanol were added to the suspension and this was left at -20ºC for at least 30 minutes. The mixture was centrifuged for 20 minutes at 13500 rpm, at 4ºC, and the supernatant discarded. The DNA pellet was washed with 70% ethanol, centrifuged for 20 minutes at 13500 rpm, at 4ºC, dried in a speed vacuum for 15 minutes and finally eluted in 50 µL of water.

IV.3.2.3. Amplification of barcodes

Amplification of the barcodes to allow unequivocal identification of the *C. glabrata* mutants was carried out using two PCR reactions, the first to amplify the barcodes of each mutant and the

second to insert the Illumina adapters required for the sequencing and the specific multiplex indexes used to distinguish between replicates and between growth conditions. A schematic representation of the overall procedure utilized is depicted in Figure IV.2. Briefly, *C. glabrata* deletion strains were engineered by double homologous recombination using a *NAT1* cassette containing two variable sequences (barcodes) while the remaining cassette sequence was identical between all deletion mutants. The first PCR reaction aimed to amplify the upstream and downstream barcodes of the deletion cassette, specific for each deletion mutant, using for that two primers specifically designed for the upstream (PU1 and PU2) and downstream (PD1 and PD2) barcode and that sit on the conserved sequences of the *NAT1* cassette (in specific they site on the U1/D1 sequences and the *NAT1* gene shown in Figure IV.2)(Table IV.1). For this 1st PCR reaction was used a mixture containing: 10 µL HF buffer; 1 µL dNTPs [10mM]; 2,5 µL PU1 primer forward [corresponding to 10 µM]; 2,5 µL PU2 primer reverse [corresponding to 10 µM]; 150 ng of DNA (1 µL); 1 U Phusion polymerase; 32,5 µL of H_2O; For the amplification of the downstream barcode was used: 10 µL HF buffer; 1 µL dNTPs [10 mM]; 2,5 µL PD1 primer forward [10 µM]; 2,5 µL PD2 primer reverse [10 µM]; 150 ng of DNA (1 µL); 1 U Phusion polymerase; 32,5 µL of H_2O. The PCR programs used for the amplification of barcodes in the 1st PCR is shown in Table IV.3. The obtained PCR products were purified by ethanol precipitation, as before detailed, and eluted in 20 µL of water.

Table IV.1. List of primers used for upstream and downstream barcode amplification (1st PCR).

Primer	Sequence
PU1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCGCTGCTAGGCGCGCCG
PU2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGACTGTCAAGGAGGGTAT
PD1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCATGCGTCAATCGTATGTG
PD2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCAGGGATGCGGC

The second PCR was performed to add to the PCR product obtained in the 1st PCR the adapters required for Illumina sequencing, as well as unique indexes for multiplex analysis. These unique indexes allowed us pool together DNA samples obtained from different technical replicas and from the two growth conditions used (single culture of *C. glabrata* or co-cultivation with *L. gasseri*). The primers used for this second PCR reaction were designed to anneal with a sequence that was specifically introduced for this purpose in the 1st PCR reaction (Figure IV.2) and that are described in Table IV.2, along with the multiplex index sequence.

Table IV.2. List of primers used for adding the Illumina adapter (required for sequencing) and the multiplex indexes to the upstream and downstream barcodes (2nd PCR).

Condition	Primers	Sequence	Multiplex Index
Single-culture	Replicate 1 FW	AATGATACGGCGACCACCGAGATCTACACagcttgatacTCGTCGGCAGCGTC	agcttgatac
	Replicate 1 REV	CAAGCAGAAGACGGCATACGAGATaagaggagcaGTCTCGTGGGCTCGG	aagaggagca
	Replicate 2 FW	AATGATACGGCGACCACCGAGATCTACACcacccactaaTCGTCGGCAGCGTC	cacccactaa
	Replicate 2 REV	CAAGCAGAAGACGGCATACGAGATctcgaattccGTCTCGTGGGCTCGG	ctcgaattcc
	Replicate 3 FW	AATGATACGGCGACCACCGAGATCTACACgttatggcagTCGTCGGCAGCGTC	gttatggcag
	Replicate 3 REV	CAAGCAGAAGACGGCATACGAGATggatggttcaGTCTCGTGGGCTCGG	ggatggttca
	Replicate 4 FW	AATGATACGGCGACCACCGAGATCTACACatgtacgcacTCGTCGGCAGCGTC	atgtacgcac
	Replicate 4 REV	CAAGCAGAAGACGGCATACGAGATacgtgtgaagGTCTCGTGGGCTCGG	acgtgtgaag
Co-culture	Replicate 1 FW	AATGATACGGCGACCACCGAGATCTACACgggaaggtgtTCGTCGGCAGCGTC	gggaaggtgt
	Replicate 1 REV	CAAGCAGAAGACGGCATACGAGATtgaccactcgGTCTCGTGGGCTCGG	tgaccactcg
	Replicate 2 FW	AATGATACGGCGACCACCGAGATCTACACttcccgatcTCGTCGGCAGCGTC	ttccccgatc
	Replicate 2 REV	CAAGCAGAAGACGGCATACGAGATtcgcggacttGTCTCGTGGGCTCGG	tcgcggactt
	Replicate 3 FW	AATGATACGGCGACCACCGAGATCTACACgtggataagaTCGTCGGCAGCGTC	gtggataaga
	Replicate 3 REV	CAAGCAGAAGACGGCATACGAGATcttggattgaGTCTCGTGGGCTCGG	cttggattga
	Replicate 4 FW	AATGATACGGCGACCACCGAGATCTACACtcgacaacatTCGTCGGCAGCGTC	tcgacaacat
	Replicate 4 REV	CAAGCAGAAGACGGCATACGAGATttaggactgtGTCTCGTGGGCTCGG	ttaggactgt

The mixtures used for this second PCR were identical to those used for the 1st PCR and the program of amplification is shown in Table IV.3. The PCR products obtained after the second PCR were excised from the agarose gel and subsequently purified using the NZYGelPure kit from Nzytech. To assess their concentration and purity before sequencing, the PCR products obtained from the seven tested pools were analysed in a DNA fragment analyser at Instituto Gulbenkian de Ciência (IGC) in Oeiras, Portugal. The 7 samples were sent for sequencing as a paid service at CD Genomics (USA).

Table IV.3. Thermocycler program used for amplification of *C. glabrata* mutant upstream and downstream barcodes (1st PCR) and addition of Illumina adapters and multiplex indexes (2nd PCR).

	Step	Temperature [°C]	Time
1x	Initial Denaturation	98	30 s
30x	Denaturation	98	10 s
	Annealing	62 (1st PCR) / 61 (2nd PCR)	10 s
	Elongation	72	5 s
1x	Final Elongation	72	5 min
1x	Hold	10	∞

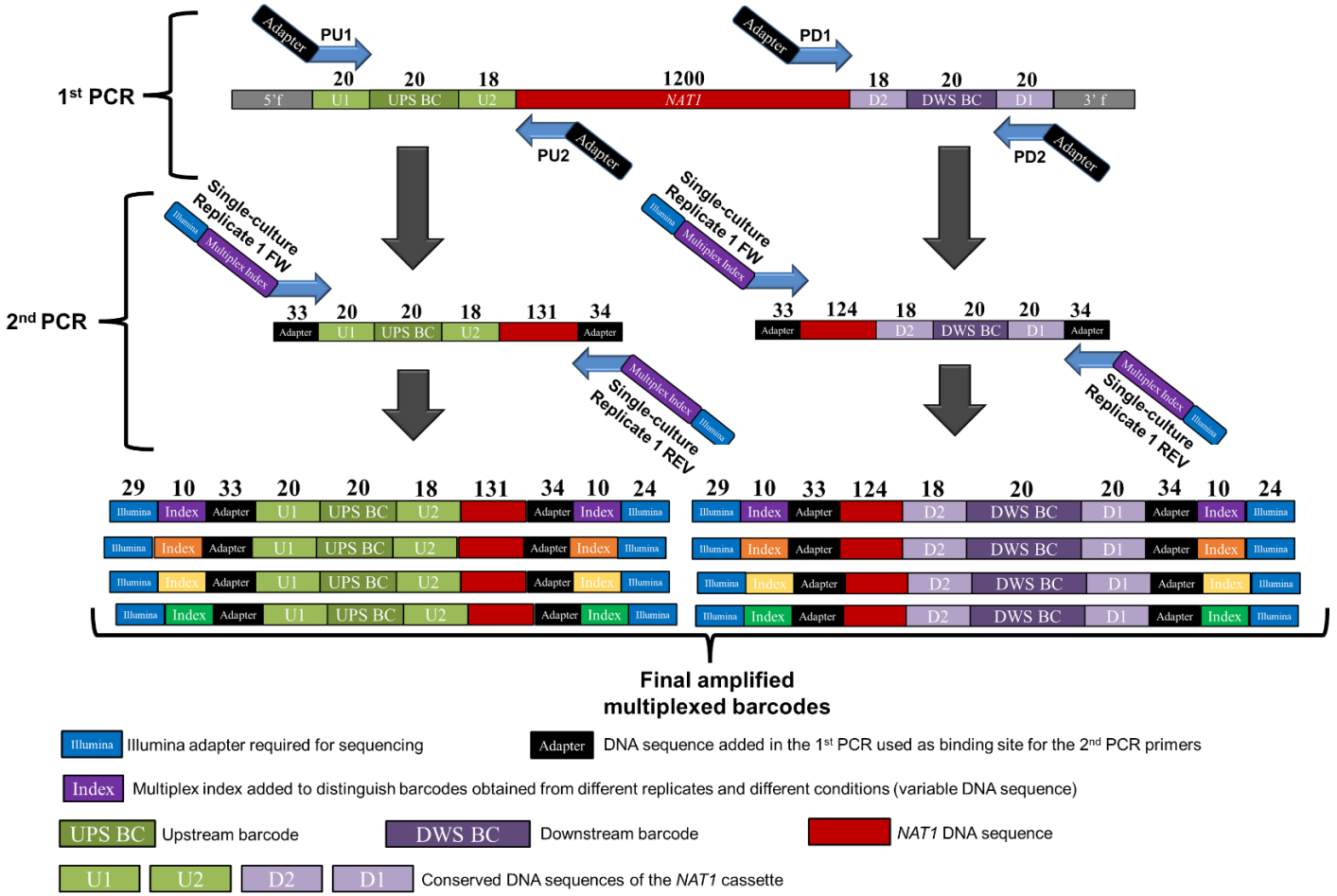


Figure IV.2. Schematic representation of the PCR strategy used for the amplification of the upstream and downstream barcodes of each single deletion mutant of *C. glabrata*. First PCR was used to amplify the barcodes and add an adapter to be served as binding site for the second PCR (black square). In the second PCR was added the Illumina adapter required for sequencing (blue square) and a multiplex index to distinguish between the DNA obtained from different replicates and different conditions (variable colour). U1, U2, D2, D1 and NAT1 sequences are conserved between all mutants. In dark green and dark grey are represented the upstream and downstream barcodes (UPS BC and DWS BC), respectively. Primer sequences are described in Table IV.1 and Table IV.2.

IV.3.3. Phenotyping of the *C. glabrata* mutant collection along co-cultivation in mixed biofilms with *L. gasseri*

To phenotype the mutant collection in co-cultivation with *L. gasseri* in sessile conditions each 96-multiwell plate containing the mutant strains was thawed and used to inoculate (using a 96-multiwell pin replicator) a new 96-multiwell plate containing 200 µL liquid YPD supplemented with 100 mg/L nourseothricin. To each plate, it was added the corresponding wild-type strain (ATCC2001 HTL or ATCC2011_Δ*his3*). The strains were afterwards incubated overnight at 30ºC using an agitation of 250 rpm. In this first day, *L. gasseri* cells were also thawed and cultivated overnight in liquid MRS at 37ºC with an agitation of 100 rpm. On the next day, the *C. glabrata* strains were resuspended, the OD_{600nm} of each well was measured and the average OD_{600nm} calculated. Using that average OD_{600nm} of the strains, a standardized volume (identical to all the strains) of each cell suspension was calculated and transferred (using a multichannel pipette) into two new 96-multiwell plates aiming to obtain a cell suspension having an OD_{600nm} of 0.1. In order to allow the establishment of the mixed biofilm one of these prepared Elisa plates was inoculated with a volume of *L. gasseri* cells (obtained by centrifugation from the pre-inoculum) that yield an OD_{600nm} of 0.2 in each of the wells. A similar Elisa plate was prepared but using only MRS without bacterial cells (this was the control). Each well contained, thus, 190 µL of fresh MRS medium or fresh MRS containing 0.2 OD_{600nm} of *L. gasseri*. Both these two Elisa plates were inoculated with 10 µL of a standardized inoculum of the different *C. glabrata* strains. This experimental setup had been previously used with success in Chapter II to examine mixed biofilms formed between *C. glabrata* and *L. gasseri*. The plates, either containing the mixed biofilms formed between the different *C. glabrata* strains and *L. gasseri*, or containing the single-species *C. glabrata* biofilms, were incubated at 37ºC, with an agitation of 25 rpm, for 24h. Cellular viability in the single- and in the multi-species biofilm was determined by fluorescence spectroscopy, after a prior labelling with propidium iodide. Briefly, after the incubation step the culture medium was removed from each of the wells, and the formed biofilm carefully washed with 200 µL phosphate-buffered saline (PBS) 1% to avoid biofilm disruption. Afterwards, 100 µL of PBS 1% containing 10µM propidium iodide were added to each of the wells and the plates were incubated, in the dark, for 30 minutes, at room temperature. After this, the supernatant was removed, the PI-labelled biofilm cells were washed with 200 µL of PBS 1% and fluorescence was measured (at an excitation of 530nm and emission of 630nm) using a multi-mode microplate reader FilterMax F5 and the software SoftMax Pro 6.2.1.

IV.3.4. Confocal imaging of mixed biofilms formed between *L. gasseri* and *C. glabrata*

The formation of mixed biofilms formed between a selected set of *C. glabrata* mutants (Δ*CCH1*, Δ*CAGL0D03674g*, Δ*CAGL0A03586g*, Δ*BCY1*, Δ*CAGL0G06754g*, Δ*CAGL0K12078g*, ΔCAGL0E02915g, Δ*CAGL0L10956g*, Δ*YPK2*, Δ*CBK1*, Δ*CAGL0B01991g*, Δ*RGT1*,

Δ*CAGL0E02805g*, Δ*CAGL0F06743g*, Δ*CAGL0L12562g*, Δ*CAGL0M03927g*, Δ*EMI1* and Δ*CAGL0F03025g*) and *L. gasseri* under the above described experimental conditions were examined by confocal microscopy. In this case, after 24h growth, the mixed biofilms were stained with 3 µM SYTO 9 for 30 min and, afterwards with 4 µM TO-PRO-3 iodide for 15 min, similarly to what was performed in Chapter II. Labelled biofilms were imaged by confocal laser scanning microscopy (CLSM) using the 488 nm Ar+ laser line (emission collected at 500 – 590 nm) for SYTO 9 imaging and the 633 nm He-Ne laser line (emission collected at 645-795 nm) for TO-PRO-3 Iodide imaging, to minimize cross-interference between the two channels. Observations were performed using a Leica TCS SP5 inverted microscope with a 63x water (1.2 numerical aperture) apochromatic objective.

IV.4. Results

IV.4.1. Design of the experimental setup to phenotype the *C. glabrata* mutant collection along co-cultivation with *L. gasseri* under planktonic conditions

OMICs analysis, in particular transcriptomics, gave valuable information regarding how *C. glabrata* and *L. gasseri* species interact with each other along co-cultivation in MRS, including the identification of a few of *C. glabrata* genes that help the yeasts thrive under such conditions, as detailed in Chapter III. To expand knowledge on this issue, a high throughput profiling of the available collection of *C. glabrata* mutants while growing in the presence of *L. gasseri* was undertaken. Although, as described in the introduction, this mutant collection had been previously profiled in the presence of other environmental stresses[644], the fact that herein we are anticipating to use it in the presence of other microbial cells posed an important challenge because it had not been performed before. Quantification of strain abundance was, as usual in this type of assays, based on sequencing of a set of barcodes present upstream and downstream of the resistance marker *NAT1* gene, used to undertake gene deletion[644]. In particular, each deletion cassette consists of the resistance marker (the *NAT1* gene), four conserved regions (U1, U2, D1 and D2) and a unique barcode that was specifically added to the genetic background of each deletion mutant allowing its unequivocal identification (Figure IV.*2*). The design of our screening was performed taking into consideration the work of David G. Robinson *et al.* which demonstrated that using an experimental design of fourfold biological replicates and 6 million reads per condition, it was possible to accurately detect significant differences while phenotyping the entire *S. cerevisiae* mutant collection that includes around 5000 strains (while ours is considerably smaller as it includes only 578 strains)[645]. The authors came to the conclusion that to reliably profile the deletion strains at least 1397 sequence reads per mutant, per condition, or 349 reads per biological replicate library, would be needed[645]. We designed our assay to obtain around 6500 sequence reads per mutant, per condition, which was well within the parameters previously established for successful barcode quantification in *S. cerevisiae*[645-647].

For that, we pooled the 578 mutants of the collection in groups of ~96 mutants that were cultivated (in quadruplicates) in two conditions (in single and in co-culture with *L. gasseri*). To amplify the barcodes we have used the strategy shown in Figure IV.3 that was designed in order to allow also the pooling of the samples into the same sequencing runs (being the experimental conditions and replicas distinguishable based on the multiplex indexes as detailed in Table IV.2).

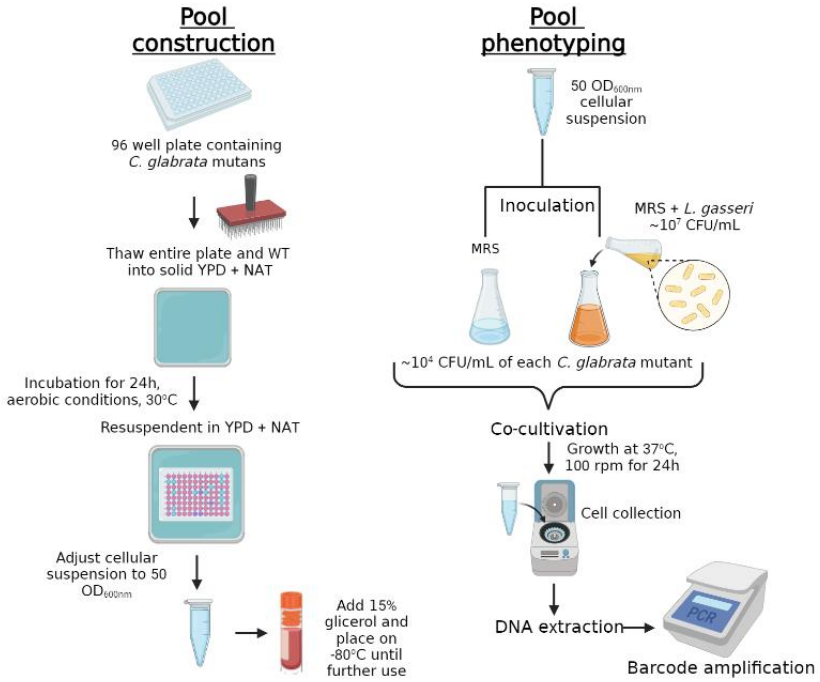


Figure IV.3. Experimental design used for the competitiveness screening, in planktonic conditions, of the *C. glabrata* mutant collection in the presence of *L. gasseri*. Individual mutants were pooled and stored until further use. Afterwards, the pooled mutants were phenotyped by their ability to grow in the presence of *L. gasseri* based on the abundance of the unique barcodes in each mutant strain that was determined by Illumina sequencing (barcode sequencing).

With this approach, the final amplicon to be sequenced encompassed around 320 base pairs including the Illumina adapters, the multiplex indexes that allowed the distinction of the replicas and experimental condition and the specific mutant barcode. In Figure IV.4 it is shown an example of the amplification products obtained after the 1st and 2nd PCR, these being subsequently purified from the agarose gel, checked for their integrity in a DNA fragment analyser and sent for sequencing.

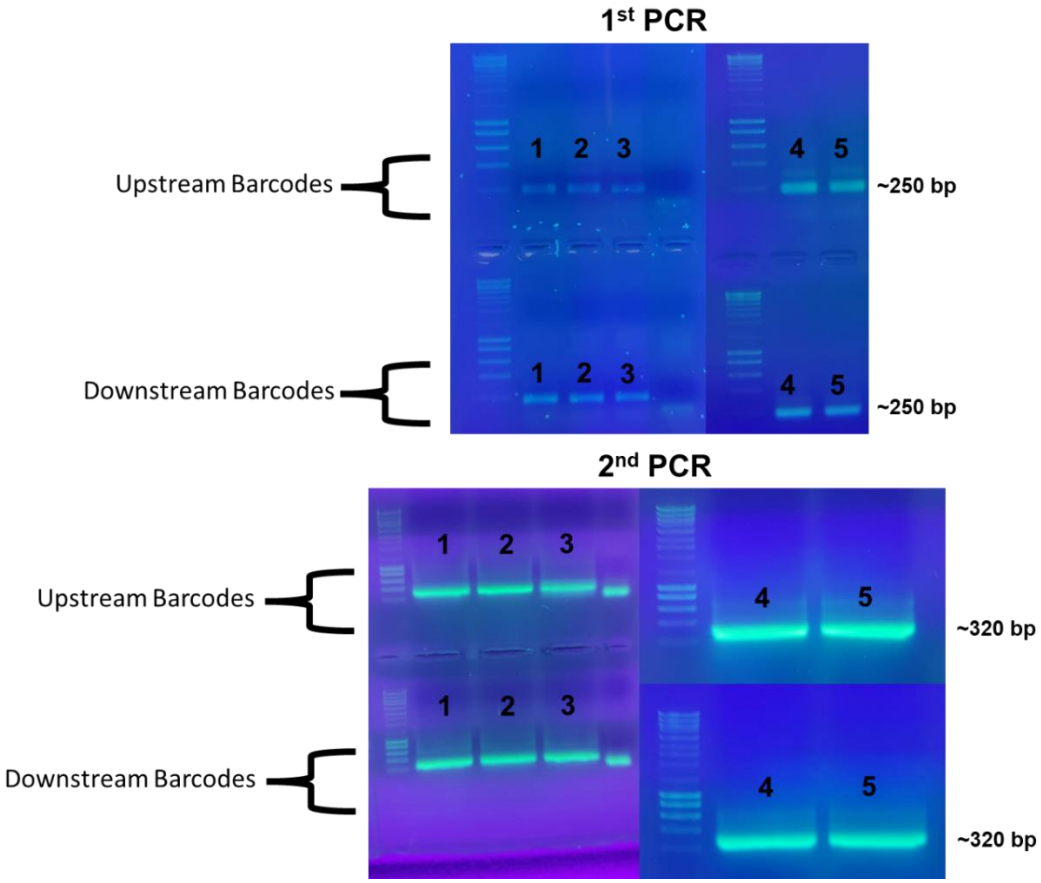


Figure IV.4. PCR strategy used for barcode amplification of the mutants belonging to the five pools constructed using the background strain HTL. The remaining two pools constructed using the Δ*his3* background strain were excluded due to a sequencing artefact. This figure shows only one replicate. The 1st PCR was used to amplify both upstream and downstream barcodes. While the 2nd PCR was performed to add the Illumina adapters and specific multiplex indexes useful for pooling various samples together. The list of primers used is shown in Table IV.1 and Table IV.2.

IV.4.2. Identification of *C. glabrata* genes required for growth in the presence of *L. gasseri* in planktonic conditions

In Figure IV.5 we are showing the results obtained for the total of *C. glabrata* mutant strains phenotyped for their growth along co-cultivation with *L. gasseri*. Strikingly, all the strains derived from the *C. glabrata* ATCC2001 (Δ*his3*) background could not be quantified since the reads obtained from these mutant pools could not be aligned with the barcodes predicted for reasons that we could not understand and that appear to have resulted from a problem while preparing and/or sequencing the DNA library. After having concluded the sequencing of the barcodes we came across the information that the initial information that we had concerning the association between barcodes and mutant strains (provided in the paper of Tobias Schwarzmüller *et al.* 2014[644]) was wrong. While examining the correct set of associations (kindly provided to us by Dr Sascha Brunke, from Hans Knoell Institute Jena (HKI), Germany) we realized that for 227 mutants we could not make a reliable quantification since we had pooled in the same cohort of mutants strains that had an identical barcode (these mutants being identified in the Annex Table IV.3). Thus, in total, only 210 out of the 578 mutants could be phenotyped properly under

planktonic conditions, this corresponding to ~36% of the overall collection. The data shown in Figure IV.5 shows a wide dispersion of barcode abundance, being clearly identifiable strains with much higher abundance than the wild-type (plotted on the top of the graph) and others clearly less abundant (plotted on the bottom of the graph). These mutant strains that exhibited reduced (below 30%) or higher (above 50%) the abundance of wild-type cells in single-culture are detailed in Annex Table IV.4, these being strains that exhibit either a poor or enhanced growth under the experimental conditions that were established (growth in MRS, 30ºC, 100 rpm for 24h). All these mutants (with higher or lower barcode abundance in single cultivation when compared with the wild-type) showed a similar behaviour in the co-culture setting. However, since this phenotype could not be attributed to the presence of *L. gasseri* (but rather to a growth defect/enhancement in MRS medium) we discarded from downstream analyses.

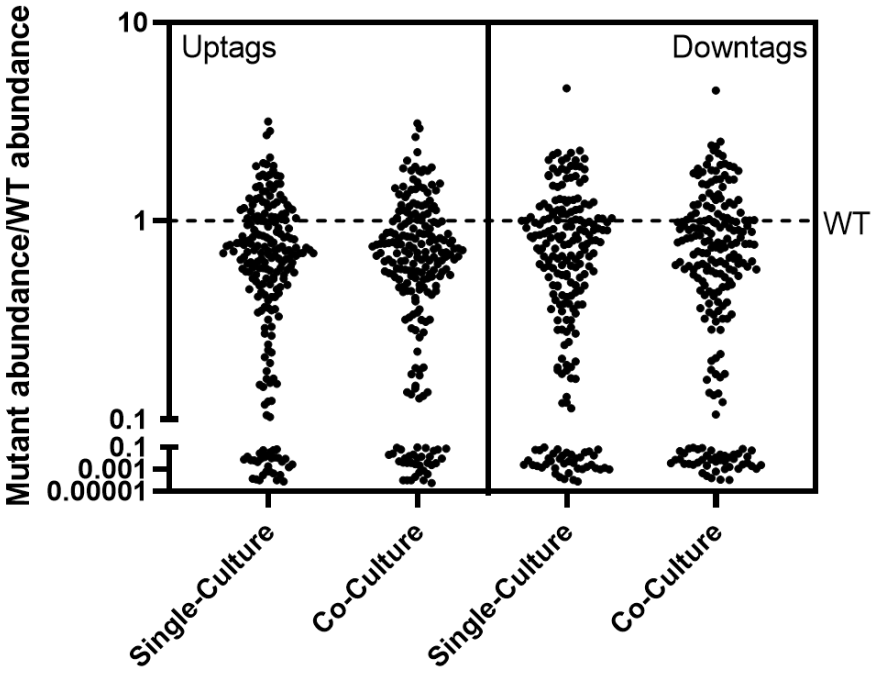


Figure IV.5. PhenOMIC analysis of *C. glabrata* deletion mutant collection. *C. glabrata* deletion mutants were grown in the absence and presence of *L. gasseri* at 37ºC and 100 rpm for 24 hours. Afterwards, the viability of each mutant was determined using their relative barcode abundance (compared to the WT). This data was obtained from 4 individual replicates for both the upstream and downstream barcode of each mutant. In total 210 single deletion mutants were properly phenotyped.

The set of mutant *C. glabrata* strains that exhibited a differential level of abundance compared to the WT strain in the presence of *L. gasseri* (while exhibiting similar abundance levels while in single-culture) is shown in Figure IV.6 and totals 7 strains. Among these were two mutants Cg∆*GLO1* and Cg∆*CAGL0G09757g*, that exhibited better growth than the parental strain, with the abundance increasing between 50% and 70%, respectively), as detailed in Figure IV.6. The genes deleted in these mutants included a lactoylglutathione lyase (*GLO1*) and a transcription factor with unclear function (*CAGL0G09757g*). On the other hand, Cg∆*CAGL0G08844g*, Cg∆*CAGL0F08833g*, Cg∆*EPA1*, Cg∆*CAGL0M05929g*, and Cg∆E*RG5* mutants were found to have increased susceptibility to *L. gasseri* compared with the wild-type (38%, 34%, 16%, 15%

and 9% less barcode abundance in co-cultivation, respectively). These less competitive deletion strains are devoid of a transcription factor with unknown activity (*CAGL0G08844g*), of two adhesins (*CAGL0F08833g* and *EPA1*), of a predicted mitochondrial matrix protein (*CAGL0M05929g*) and of the putative C22 sterol desaturase (Erg5). In Figure IV.6 are shown how the abundance of these strains in the co-cultures with *L. gasseri* changed in comparison with the one of the parental strain zooming in on the results shown in Figure IV.5 for the overall set of strains.

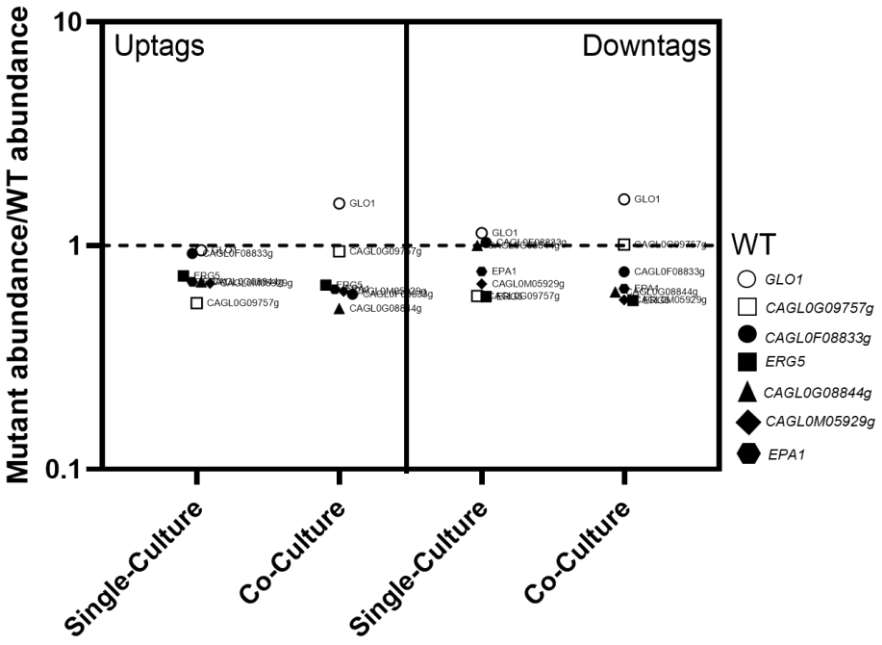


Figure IV.6. *C. glabrata* genes required for competitiveness against *L. gasseri* in biofilm forming conditions. Growth was performed at 37ºC and 100 rpm for 24 hours. This data was obtained from 4 individual replicates for both upstream and downstream barcodes of each mutant. Competitiveness genes were identified based on their relative abundance in single cultivation (similar to the WT) and in co-cultivation with *L. gasseri* (more or less barcode abundance compared with the WT) using one-way ANOVA. Each dot corresponds to a single deletion strain. Open symbols represent the genes with enhanced competitiveness while closed symbols represent genes with decreased competitiveness.

IV.4.3. Identification of *C. glabrata* genes required for growth in the presence of *L. gasseri* in biofilm forming conditions

To identify the *C. glabrata* genes required for the ability of this yeast to thrive in a mixed biofilm with *L. gasseri* we had to design a new experimental setting since all the work already published (and that we have also leveraged on while profiling the collection in planktonic conditions) did not used sessile conditions, let alone in a multi-species setting. A schematic representation of the protocol herein developed is provided in Figure IV.7. The method was based on the use of propidium iodide (PI), that is a well described marker of cellular viability due to its inability of permeate cells with intact plasma membrane[648]. Differently, in non-viable cells, PI intercalates between the DNA bases (without any sequence preference) and it can be detected using an excitation wavelength of 490nm and an emission of 630nm. The results in Chapter II

show that during formation of mixed biofilms between *L. gasseri* and *C. glabrata* there is a significant reduction in the viability of the yeast cells while the bacterial cells remain largely alive (Chapter II). Thus, we hypothesized that using PI fluorescence we could measure the loss of viability of the yeast cells, but not of the bacterial ones. Based on this hypothesis, each mutant was cultivated individually in each well of 96 well ELISA plates in single cultivation or in co-cultivation with *L. gasseri*.

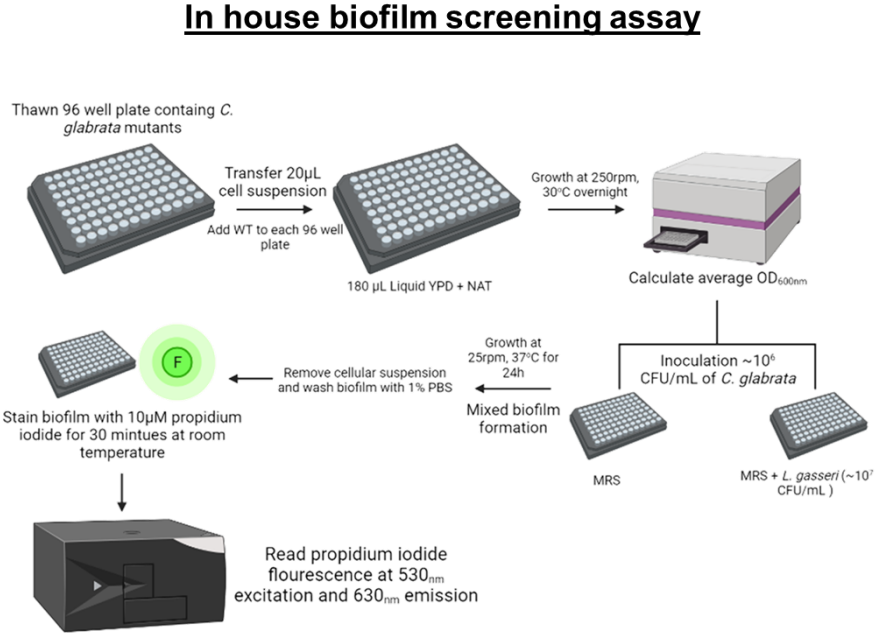


Figure IV.7. Experimental design used for the competitiveness screening, in biofilm forming conditions, of the *C. glabrata* mutant collection in the presence of *L. gasseri*. Each mutant was grown individually in the presence and absence of *L. gasseri*, and their competitiveness was assessed based on their viability determined using the fluorescence dye propidium iodide (fluorescence spectroscopy).

With this experimental setting we could find differences in the PI-labelling of a cohort of mutants that we have profiled as a proxy of what would happen while profiling the entire mutant collection. In this preliminary screening, we could identify mutant *C. glabrata* strains that accumulated higher levels of the fluorophore, suggestive that these strains would exhibit a higher number of dead cells when in a mixed biofilm with *L. gasseri*, compared to *C. glabrata* cells of the parental strain. To assure that was the case, a validation of the assay was performed in which a selected set of these mutants, selected based on their levels of accumulation of PI in the high-throughput assay, were imaged while in a biofilm with *L. gasseri* using confocal fluorescence microscopy, as done in Chapter II. The results of that imaging of the biofilm provided by confocal microscopy is in Figure IV.8 showing clearly that co-cultivation of wild-type *C. glabrata* cells with *L. gasseri* resulted in a partial loss of viability of the population of *C. glabrata* higher than the one registered in the biofilm formed by this species alone (Figure IV.8 panel A). These results recapitulate and confirm those obtained before in Chapter II. For all the deletion strains tested, a similar decrease in viability in the mixed biofilms, compared with the biofilms while in single-culture, was obtained (Figure IV.8 panel A), albeit at different levels. These results confirming the value of the used PI-labelling in the fast screening of more susceptible strains to *L. gasseri*. It has

however to be noted that the higher levels of PI did not necessarily correlated with higher cell death of the yeast cells since Δ*CCH1* was the strain showing the lowest increase of PI compared to the levels exhibited by cells of the parental strain, but it was the strain in which the loss of viability was more prominent (Figure IV.8 panel B). It has to be pointed out that the surface in which these biofilms were formed was not exactly the same; in the initial PI-based screening (that used polystyrene Elisa plates); and in the confocal microscopy assays was used µ-slide 8 well plates (e.g., µ-slide plates have a flat surface while the Elisa plates have a well like shape surface) which can underlie some of the differences found. Either way, these results showed us that the initial PI-based screening could be useful to identify among the large pool of available *C. glabrata* mutant to test those that could be more susceptible *to L. gasseri* while in a mixed-biofilm, despite it is still necessary to make confirmatory tests with confocal imaging to get more definitive conclusions on the reduction of cellular viability.

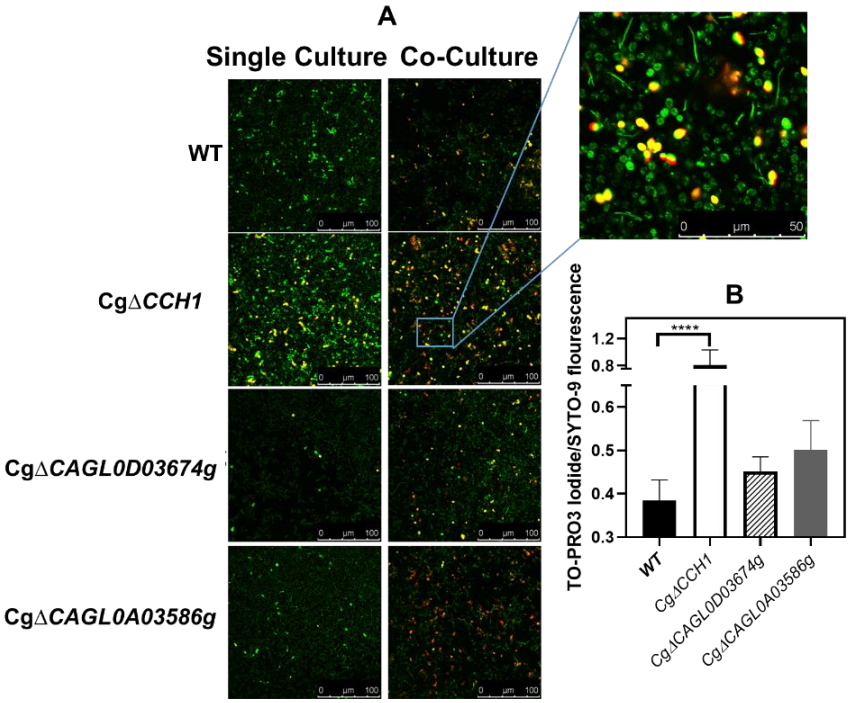


Figure IV.8. (A) Live/dead imaging of cells in single-species or mixed biofilms formed by *L. gasseri*, *C. glabrata* WT, Cg∆*CCH1*, Cg∆*CAGL0D03674g* and Cg∆*CAGL0A03586g* after 24h of cultivation, at 37ºC and 25 rpm, in MRS. The insert details the labelling of the dead yeast cells while in the mixed biofilms. The images presented are representative of a set taken from the biofilms from three replica experiments; *Candida* spp. single and mixed biofilms scale bar corresponds to 100 µm while the insert scale bar corresponds to 50 µm; **(B)** Quantification of the number of dead *Candida* cells in the multi-species biofilms formed, based on quantification of the number of red-labelled yeast cells in all pictures taken from the biofilms, compared to the total number of cells in the field (corresponding to green-labelled cells). For this quantitative analysis, more than 1000 cells were imaged in each condition. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

Reassured by the results of this preliminary screening, we undertook the profiling of the remaining *C. glabrata* mutant collection resulting in phenotyping of all 578 strains (Figure IV.9). In the background of ATCC2011 no significant differences in PI labelling were observed between the mutants and the cells of the parental strain when forming biofilms in the absence of *L. gasseri* (Figure IV.9). In the case of mutants derived from ATCC2011_∆*his3* background such differences were observed, with mutants Cg∆*INO2*, Cg∆*RTG1*, Cg∆*CAGL0L05412g*, Cg∆*SNF5* and

Cg∆*CAGL0F06237g* showing increased PI-fluorescence, comparing to the levels exhibited by the parental strain cells (Figure IV.9). The genes lacking in these mutants encode, respectively, a transcriptional regulator involved in inositol biosynthesis (CgIno2), a transcription factor involved in osmotic stress response (CgRtg1), CgSnf5 involved in alternative carbon source utilization under hypoxia and the poorly characterized proteins CAGL0L05412g and CAGL0F06237g. Further studies will be required to confirm if indeed the viability of these mutants in single-species biofilms is lower than the one of the parental strain and the molecular reasons underneath that phenotype.

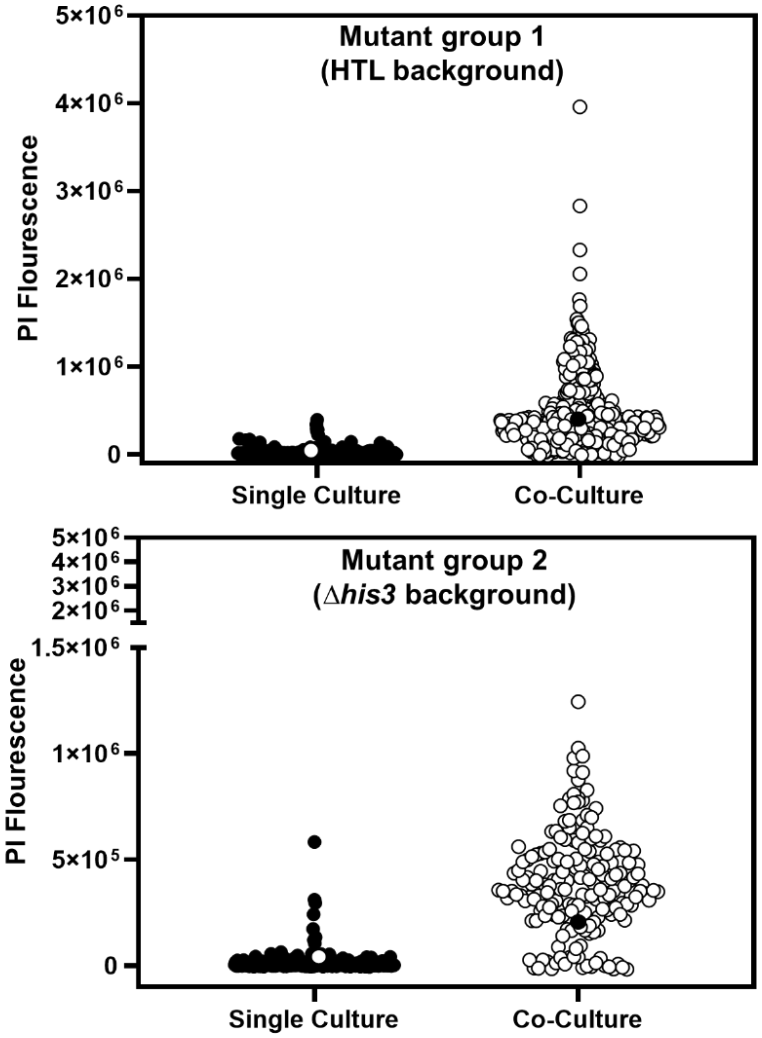


Figure IV.9. Susceptibility of *C. glabrata* mutant strains to the presence of *L. gasseri* in biofilm forming conditions. Growth was performed at 37ºC and 25 rpm for 24h to allow biofilm formation. Each dot corresponds to a single deletion strain. Mutants were divided into two groups based on the background strain used to delete the respective gene. Mutant group 1 was built in the HTL strain, while mutant group 2 was built in the background strain ∆*his3*. The respective WT strain for each group is indicated with the contrary colour dot. This data was obtained from five different replicates. In total were phenotyped 578 mutant strains of *C. glabrata* for their ability to survive in the presence of *L. gasseri*. Susceptibility was determined based on the propidium iodide fluorescence of each strain.

In the presence of *L. gasseri* a wider distribution of fluorescence values was obtained, compared to the one observed in single-species biofilms, reflecting what can be a significantly wider impact of the bacteria. After downstream analysis of the results, we identified 77 strains as

having higher levels (above 2.5-fold) of PI fluorescence in the mixed biofilms formed with *L. gasseri*, compared to those exhibited by the parental strain (shown in Figure IV.10 and Annex Table IV.5). This higher accumulation of PI suggests that the genes missing in these strains could be required to increase survival of *C. glabrata* in the mixed biofilm. In Figure IV.10 we provide a more detailed analysis of how their levels of fluorescence differed from those of the wild-type strain, while in Table IV.4 we provide a list of some of these genes (the full list is available in Annex Table IV.5).

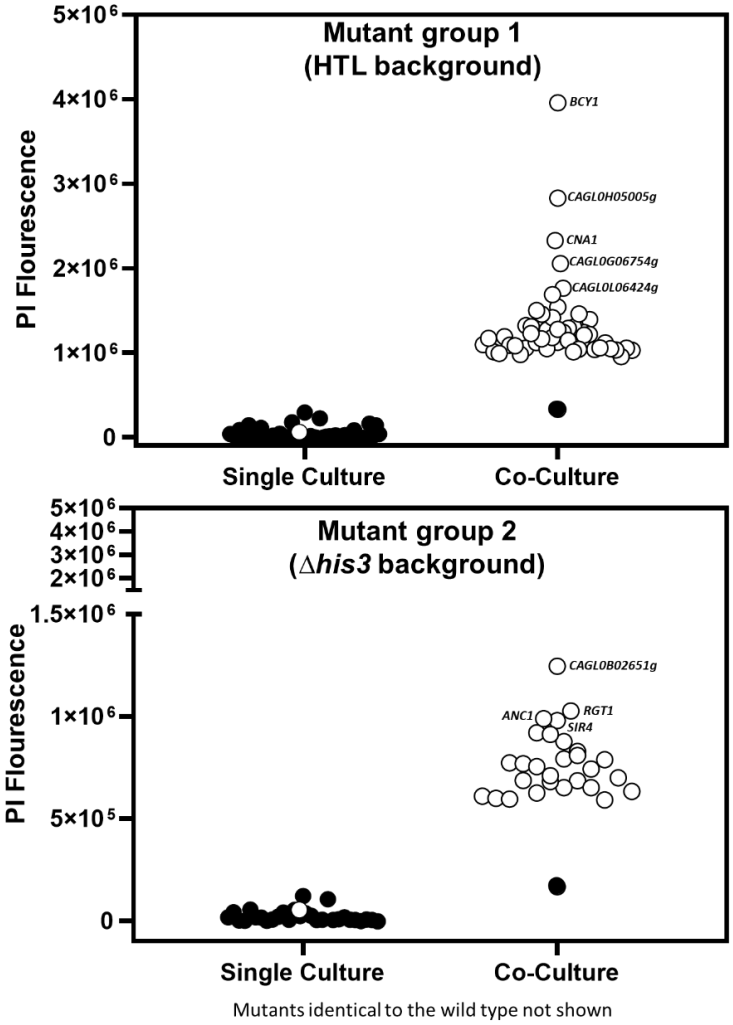


Figure IV.10. Susceptible *C. glabrata* mutant strains to the presence of *L. gasseri* in biofilm forming conditions. Growth was performed at 37ºC and 25 rpm for 24h to allow biofilm formation. Each dot corresponds to a single deletion strain. Mutants were divided into two groups based on the background strain used to delete the respective gene. Mutant group 1 was built in the HTL strain, while mutant group 2 was built in the background strain Δ*his3*. The respective WT strain for each group is indicated with the opposite colour dot. This data was obtained from five different replicates. Susceptibility was determined based on the propidium iodide fluorescence of each strain. The list of the susceptible *C. glabrata* deletion strains in co-cultivation with *L. gasseri* is described in Annex Table IV.5.

Table IV.4. Selected strains of *C. glabrata* with increased susceptibility to the presence of *L. gasseri* in biofilm forming conditions. Growth was performed at 37ºC and 25 rpm for 24h. Table describes the deleted ORF, the gene name when available, the *S. cerevisiae* ortholog, the decreased viability fold change compared with the WT and a brief gene description obtained from the *Candida* genome database (CGD). The remaining susceptible genes are described in Annex Table IV.5.

Deleted ORF	Gene Name	*S. cerevisiae* ortholog	PI fluorescence (fold change)	Gene Description (CGD)
CAGL0I05236g	*BCY1*	*BCY1*	12.0	cAMP dependent protein kinase, regulatory subunit
CAGL0H05005g		*CSR1*	8.6	Ortholog(s) have phosphatidylinositol transfer activity
CAGL0L11110g	*CNA1*	*CMP2*	7.1	Catalytic subunit of calcineurin, calcium/calmodulin-dependent Ser/Thr-specific protein phosphatase; regulates stress-responding transcription factor Crz1p; involved in thermotolerance, response to ER stress, cell wall integrity, virulence
CAGL0G06754g		*CDC10*	6.2	Ortholog(s) have 1-phosphatidylinositol binding, GTPase activity, molecular adaptor activity, phosphatidylinositol-4-phosphate binding, phosphatidylinositol-5-phosphate binding, structural constituent of cytoskeleton activity
CAGL0M03597g	*MID1*	*MID1*	4.3	Putative calcium transporter; putative regulatory subunit of a plasma membrane gated channel involved in Ca2+ uptake; required for viability upon prolonged fluconazole stress
CAGL0M06325g		*SMP1*	4.1	Ortholog(s) have DNA binding, bending, sequence-specific DNA binding activity and cytoplasm, nucleus localization
CAGL0L05632g	*PBS2*	*PBS2*	4.0	Ortholog(s) have MAP kinase kinase activity, MAP-kinase scaffold activity
CAGL0F03003g		*HKR1*	3.9	Ortholog(s) have osmosensor activity and role in (1->3)-beta-D-glucan biosynthetic process, cellular bud site selection, fungal-type cell wall organization, hyperosmotic response, osmosensory signalling pathway via Sho1 osmosensor
CAGL0M06831g	*CRZ1*	*CRZ1*	3.6	Transcription factor; downstream component of the calcineurin signalling pathway
CAGL0F09097g	*SKN7*	*SKN7*	2.9	Predicted transcription factor, involved in oxidative stress response; required for induction of *TRX2*, *TRR1* and *TSA1* transcription under oxidative stress

To confirm the results obtained, a set of 10 mutants identified in the screening as being more susceptible to *L. gasseri* than cells of the parental strain, were selected for subsequent imaging of the mixed biofilm (labelled with TO-PRO-3 iodide and SYTO9) by confocal microscopy. The results obtained with these strains are shown in Figure IV.11. All these mutants exhibited a higher level of fluorescence than the one exhibited by the cells of the parental strain, which was a remarkable observation and again reinforced the findings obtained in the initial screening only performed with PI. Despite this, in some cases the difference obtained for the cells of the parental strain was not high enough to achieve statistically significance.

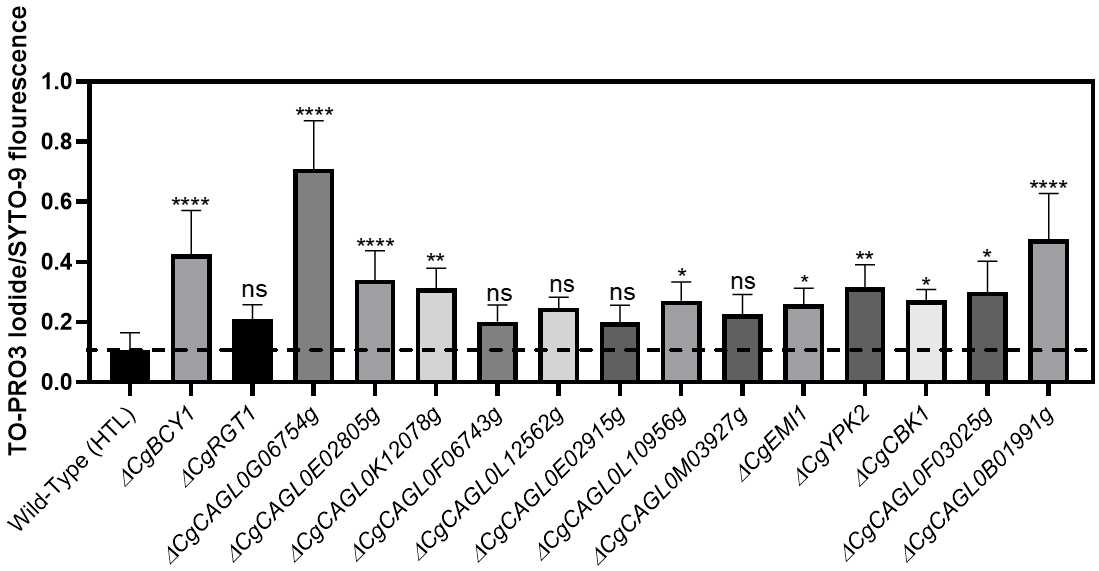


Figure IV.11. Susceptibility of *C. glabrata* deletion strains in the presence of *L. gasseri* in biofilm forming conditions using fluorescence confocal microscopy. Growth was performed at 37ºC and 25 rpm for 24h to allow biofilm formation. The results presented are representative of three independent replicas. The selection of the susceptible deletions strains to be tested was based on the screening assay using propidium iodide. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

In Figure IV.12 is shown the functional distribution of the 77 *C. glabrata* genes that we have found as being required for maximal growth of *C. glabrata* while in a mixed biofilm with *L. gasseri*. A first glimpse over the results shows that they are widespread in terms of their biological function including genes involved in adhesion, in amino acid and carbohydrate metabolism and transport, in cell signalling, in cell wall structure, in actin cytoskeleton organization, in metabolism and transport of inorganic ions, in lipid metabolism (in particular, sterol biosynthesis), among others. The biological function comprising the higher number of *C. glabrata* genes maximizing growth in the mixed biofilm with *L. gasseri* was “transcriptional regulation” and included 21 genes. Among these were the transcription factor *SKN7*, involved in oxidative stress response[649]; *HAL9*[650], involved in resistance to acidic environments; *SMP1* involved in osmoadaptation and maintenance of cell wall integrity[651, 652]; and many other, such as *CAGL0M06325g*, *CAGL0M03927g* and *CAGL0F06743g*, that are poorly characterized. A robust number of genes was also clustered in the “signalling” functional class that included key players of the PKA (*BCY1*), calcineurin (*CNA1* (catalytic subunit of calcineurin, calcineurin A) and *CRZ1* (downstream transcriptional regulator)) and HOG signalling pathway (*HKR1* (osmosensor) and *PBS2*

(MAPKK)). The *C. glabrata* genes required for maximal growth in the presence of *L. gasseri* clustered in the amino acid metabolism and transport class included the genes *CAGL0F06501g*, involved in ornithine biosynthesis; *CAGL0B02651g*, involved in methionine biosynthesis and *THI3*, involved in thiamine biosynthesis.

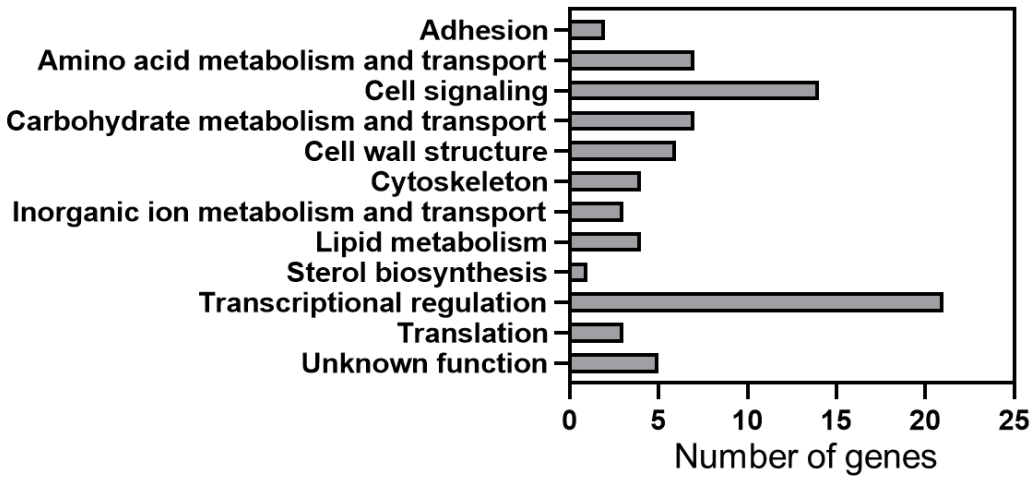


Figure IV.12. Functional analysis of the cohort of *C. glabrata* genes identified as modulators of the competitiveness of the yeast in mixed biofilms with *L. gasseri*. Functional analysis was manually curated. Among these genes, transcriptional regulation, cell signalling and amino acid and carbohydrate transport and metabolism were the biological functions with more genes.

IV.4.4. Discussion

This work focused on the identification of *C. glabrata* genes that could be mediating the competitiveness of the yeast when co-cultivated in the presence of *L. gasseri*, either in planktonic and in biofilm forming conditions. The assay conducted under sessile conditions was pioneering as it was the first of such kind described in the literature in which a mutant collection is phenotyped in a mixed biofilm setting. Although different technical problems limited the range of strains that could be tested under planktonic conditions, still this was also innovative since it is the first study focusing on the interaction of this yeast with *L. gasseri*, advancing thus what is the current knowledge on this relevant ecological interaction. In particular, in the screening performed it was not possible to phenotype the mutants derived from the Δ*his3* background and also those mutants that were pooled together although they had identical barcodes. While for the profiling of the first mutant set, the phenotypic screening can likely be performed using the same conditions herein described (as the problem appeared to have occurred at the DNA sequencing step), to phenotype the second set of mutants (built in the HTL background) it will be necessary to make new mutant pools, placing in different pools the strains that have identical barcodes. Note that these issues only affected the examination of the co-cultivation in planktonic conditions since the screening undertaken with the biofilm-forming cells were performed individually (that is without pooling the strains) and did not require DNA sequencing. Despite the difficulties, it was still possible to identify the adhesins EPA1 and *CAGL0F08833g* and the sterol desaturase Erg5, as being required for maximal growth of *C. glabrata* in the presence of *L. gasseri*. Interestingly, in the transcriptomic analysis undertaken in Chapter III we could also observe an up-regulation of *C. glabrata* genes involved in adhesion in the co-culture, including of *EPA1* (that was up-regulated 14-fold). Although we hypothesized that this up-regulation could result from the higher cell density that could somehow stimulate cell-cell contacts, the herein demonstration that these adhesins also maximize growth of the yeast is intriguing. It is possible that the lack of Epa1 could alter the cellular surface of the yeast resulting in an increased susceptibility to the compounds produced by *L. gasseri* or even the physical contact between these species. In this sense adhesins have been described to have roles other than adhesion, for example Epa3 was shown to mediating azole resistance in *C. glabrata*[653]. The identification in this cohort of *CgCAGL0F08833g* is particularly interesting considering its similarity with *S. cerevisiae ScMSB2*, a mucin family member described to be a plasma membrane sensor of the HOG signalling pathway leading to the activation of the pathway. Specifically, *MSB2* is an upstream element of the *SHO1* branch, responsible for the activation of the Hog1p kinase[636]. Considering the already demonstrated essential role of the Hog1-pathway in determining the *C. glabrata*/lactobacilli interaction[635], it is possible that the deletion of *MSB2* decreased the activity of Hog1 and, consequently, reduced growth of *C. glabrata* while in co-culture with *L. gasseri*. It will be interesting to evaluate in the future if the remaining genes of the HOG pathway are also required for survival of *C. glabrata* in the presence of *L. gasseri* since only the strains lacking the genes *SHO1*, *PBS2*, *HKR1*, *SMP1 STE20*, *STE50 and SSK1* were tested and found to be not susceptible to *L. gasseri* under the

planktonic conditions. This pathway seems to be even more relevant for survival of *C. glabrata* in mixed biofilms as it will be discussed shortly.

The deletion of C*AGL0G08844g* also reduced growth of *C. glabrata* along planktonic growth in the presence of *L. gasseri*. This gene was found to encode a transcription factor required for *C. glabrata* tolerance to acidic conditions, caused by supplementation of the medium with the strong acid HCl[650]. Previously (in Chapter II) we have shown that co-cultivation of *C. glabrata* with *L. gasseri* involves acidification of the medium due to the accumulation of lactic acid in the broth. Thus, it is possible that the registered positive effect of C*AGL0G08844g* expression can also benefit *C. glabrata* cells although the mechanisms by which lactic acid and HCl exert toxicity are very different. In the undertaken screening of the mutant collection, we have successfully profiled the effect in *C. glabrata* growth of only 5% (corresponding to 17 strains) of the genes documented to be regulated by C*AGL0G08844g* with none of these showing an effect in improving growth of *C. glabrata* while in co-cultivation with *L. gasseri*. It is expected that the conclusion of the screening can help better understand this protective effect exerted by C*AGL0G08844g* against *L. gasseri* since 27 other mutants devoid of C*AGL0G08844g* -targets will be profiled.

In the results described in Chapter III we show that the expression of *CYB5*, involved in sterol biosynthesis, improves *C. glabrata* growth in the presence of *L. gasseri*. Herein we show that *CgERG5*, encoding a C-22 sterol desaturase also involved in the ergosterol biosynthetic pathway, is also necessary for maximal *C. glabrata* growth while in co-cultivation with *L. gasseri*. It is conceivable that the deletion of *ERG* genes can affect the overall composition of the plasma membrane of *C. glabrata* cells resulting in the susceptibility of these cells to the presence of the bacteria and eventually also to the presence of metabolites they produce that can be harmful, including lactic acid. Indeed, in *S. cerevisiae* the levels of ergosterol in the plasma membrane were recently linked with higher lactic acid tolerance[654]. Also, as shown in Chapter II, along co-cultivation of *C. glabrata* with *L. gasseri* in MRS there is an expected accumulation of ~30 mM acetic acid which, although is well below the concentrations shown to exert toxicity for this species *in vitro* at this pH[430], it may pose some challenge for more susceptible strains as it can be the case of the Δ*erg* mutants above described.

To further extend the knowledge about the interaction established between *L. gasseri* and *C. glabrata* and also considering that *in vivo* these species are expected to interact in the form of a mixed biofilm, we have also phenotyped the *C. glabrata* mutant collection in sessile conditions. During this phenotyping we could detect some loss of cell viability in wild-type cells even in biofilms formed only by *C. glabrata*, which may result from the lower availability of the cells to nutrients and the accumulation of damaging compounds[655]. We could also identify a set of mutants that exhibited even lower viability than the one exhibited by cells of the parental strain in this single-species biofilms including strains devoid of genes encoding the transcription factors Ino2, Rtg1 or *CAGL0F06237g*; the histone binding protein *CAGL0L05412g* and Snf5, a

DNA translocase. Taking into consideration the little that is known concerning the reasons underlying loss of cell viability in biofilms, it is difficult to hypothesize what could be the reasons underlying their protective effect but this certainly deserve attention in future studies aiming to limit capacity of *C. glabrata* to form biofilms.

As for the genes that improved protection to *C. glabrata* while in a mixed biofilm with *L. gasseri*, we could uncover several involved in different multiple signalling pathways involved in response to environmental stress, with emphasis on the Hog1 and calcineurin-pathways (represented in Figure IV.13). The involvement of the HOG pathway was not entirely surprising, since it was before shown its involvement in the response of *Candida* to lactobacilli and associated with the presence of lactic acid[635]. In particular we found herein that the deletion of *CgHKR1* and *CgPBS2*, two upstream elements of the HOG signalling pathway, resulted in the prominent loss of viability of *C. glabrata* cells while in a mixed biofilm with *L. gasseri*. CgHkr1 is responsible for transmitting into the pathway cues from the external environment, while CgPbs2 directly phosphorylates Hog1 protein under osmotic stress, resulting in the activation of the kinase and its downstream targets[636, 656]. Notably, Sho1, the other protein that belongs to the same signalling branch as Hkr1 and Pbs2 was also more susceptible to the presence of *L. gasseri* than wild-type cells, however the difference in fluorescence was of 1.7-fold, below the threshold of 2.5 fold that we used to filter the data and thus this gene was not in the lists of susceptible mutants shown in Annex Table IV.5. The deletion of the transcription factor *CgSMP1*, a downstream target of Hog1[651, 652], also resulted in increased *C. glabrata* susceptibility to *L. gasseri*. After activation by Hog1, Smp1 induces the expression of genes involved in the osmoadaptation response and maintenance of cell wall integrity[652, 657]. Up to now there is no information concerning documented targets of Smp1 but it will be interesting in the future to identify these and, eventually, profile their growth while in a mixed biofilm with *L. gasseri*. We could also not profile in this screening the Δ*hog1* mutant because this strain was not present in the initial collection, but this is also something that should be performed to confirm the findings that the HOG pathway is indeed involved in response of *C. glabrata* to *L. gasseri* in mixed biofilms. On the overall, it seems that the *SHO1* branch of the HOG pathway is the one crucial for response crucial for the survival of *C. glabrata* in mixed cultures, although it was interesting to note that deletion *STE20* and *STE50*, downstream elements of the Sho1-pathway[636, 637], did not affected competitiveness of *C. glabrata* cells while in the mixed biofilm, meaning that signal transduction could use other proteins. It is also crucial to mention that the observations abovementioned could be related with the demonstration that deletion of Hog1 protein resulted in susceptibility of the mutant to lactic acid under physiological relevant concentrations (11 to 180mM)[635], implicating this pathway in resistance to this weak acid produced by lactobacilli. Thus, disruption of HOG pathway, through the deletion of several upstream genes, can result in the increased susceptibility of these mutants to lactobacilli, in specific susceptible to the lactic acid that is produced by *L. gasseri* in co-cultivation (of about 5 g/L in planktonic conditions) (Annex Figure II.4 panel A).

The other pathway that emerged more prominently from the screening as mediating competitiveness of *C. glabrata* in the presence of *L. gasseri* was the calcineurin signalling pathway, as shown in Figure IV.13. In *Candida* this pathway is involved in ion homeostasis, sphingolipid and cell wall biosynthesis, protein trafficking, ubiquitin, autophagy, adaptation to stress and antifungal resistance[658-661]. Calcineurin is a calcium/calmodulin-dependent serine/threonine-specific protein phosphatase with a catalytic A (Cna1) and a regulatory B calcium-binding subunit (Cnb1)[662, 663]. This pathway is activated by calcium binding to calmodulin that afterwards activates the dephosphorylation cascade that activates the transcription factor Crz1[663-665]. Deletion of the calcineurin catalytic subunit *CNA1*, of its main target, the transcription factor Crz1, and of the calcium plasma membrane transporter *MID1*, resulted in the increased susceptibility of *C. glabrata* cells to *L. gasseri* in mixed biofilms. Deletion of *CCH1*, the other calcium transporter of the calcineurin pathway[665], also reduced viability of *C. glabrata* cells while in the presence of *L. gasseri* as shown by confocal microscopy imaging of the mixed biofilms that we did. Necessarily, in the future work it will be needed to identify the set of target genes that could be under Crz1 regulation in these conditions, but among those that are already known (based on the information available in the PathoYeastract database), we observed that deletion of *CAGL0J09416g*, encoding a protein of unknown function, localized to the vacuolar membrane, also reduced viability of *C. glabrata* cells in the presence of *L. gasseri*. Interestingly, in *C. albicans*, β-glucan masking induced by lactate, was also found to be dependent of *CRZ1*[289], for which it is tempting to speculate that the eventual production of this metabolite could be inducing the pathway also in these conditions. It is also important to mention that in addition to the susceptibility that the *CRZ1* deletion mutant has when co-cultivated with *L. gasseri*, targeting this gene could also reduce the β-glucan masking allowing for a better action of the host immune defense, making it a very promising target to be used to formulate new anti-*Candida* therapies. It will be interesting to establish whether the genes mediating this β-glucan evasion mechanism also affect, somehow, the tolerance of *C. glabrata* cells to *L. gasseri*.

Other proteins of known function that also emerged from our screening as mediating tolerance of *C. glabrata* to *L. gasseri* included Bcy1, the regulatory subunit of the PKA pathway; the transcription factor Skn7 involved in oxidative stress; the predicted zinc transporters *CAGL0E01353g* and *CAGL0M08250g* involved in zinc uptake and homeostasis; as well as genes involved in adhesion (*CAGL0L06424g*, *CAGL0E02915g*) that were also shown to be required for survival in the presence of *L. gasseri* in planktonic conditions (schematically represented in Figure IV.13). The challenge for the future will be to elucidate what could be the role played by these genes in determining the interaction between *L. gasseri* and *C. glabrata* in the context of the mixed biofilm. Additionally, would be interesting to study the effect of pharmaceutical that could specifically target and inhibit these pathways, as it is for the case of Crz1 that is inhibited by FK506[666]. The use of such pharmaceuticals would shift the ecological balance in favour of the bacteria resulting in the inhibition of *Candida* from the environment.

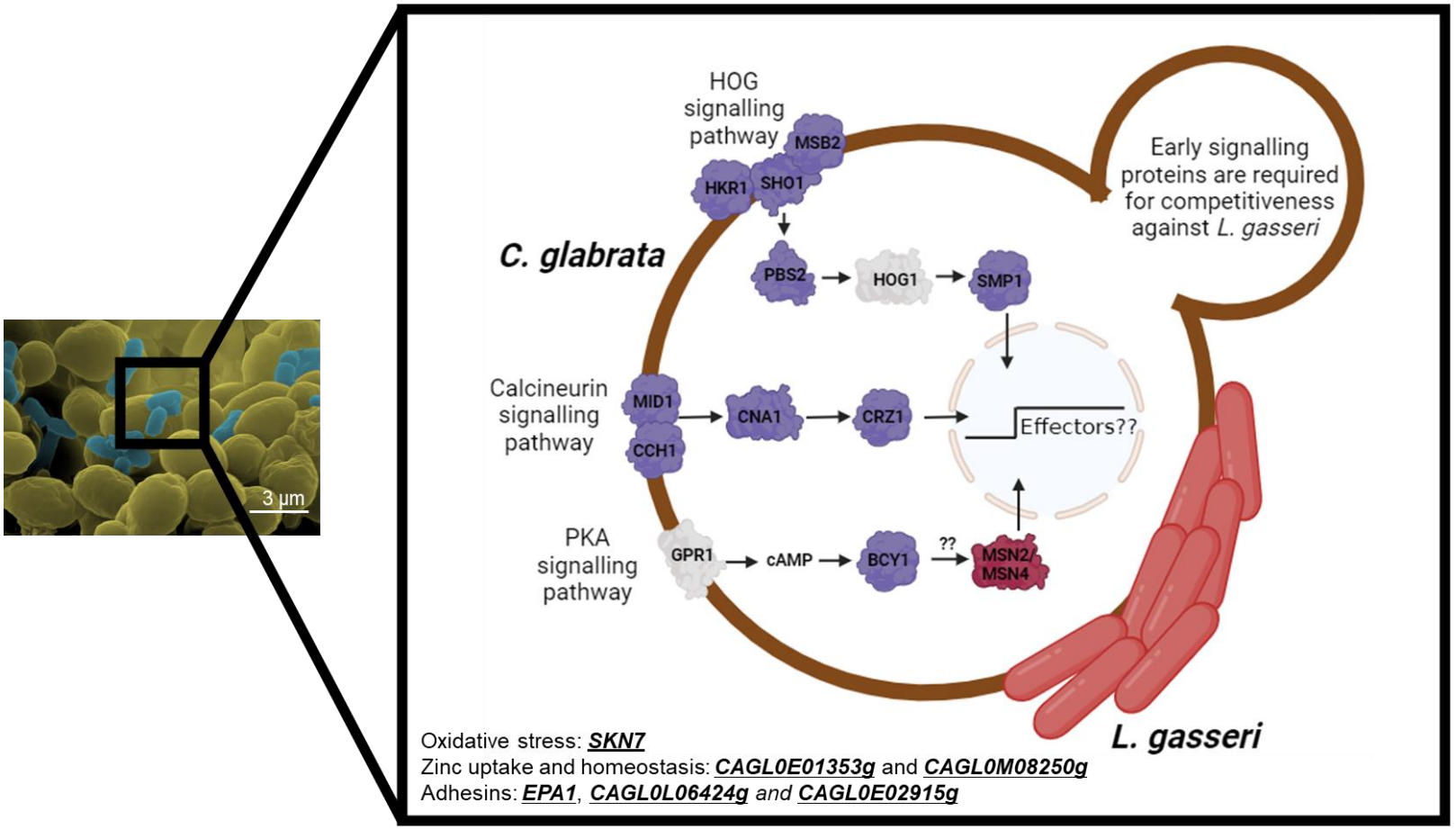


Figure IV.13. Signalling pathways found to be required for *C. glabrata* survival in the presence of *L. gasseri*. The competitiveness of *C. glabrata* against *L. gasseri* was found to be dependent on genes related to upstream proteins of the PKA, HOG and calcineurin signalling pathway. Genes in purple were phenotyped and found to be susceptible while genes in red were phenotyped but not susceptible. Genes in grey were not phenotyped because they were not part of the *C. glabrata* mutant collection. Note that all of the genes, except for *MSB2* and *EPA1* (found to be required in the planktonic conditions), were obtained from the phenotypic assay in biofilm forming conditions. Despite this, we decided to add them to the model to highlight the role of the HOG pathway and adhesins in the interaction between these species. Additionally we found *SKN7*, two zinc uptake and homestasis related transporters and two other adhesins to be required for survival of *C. glabrata* in mixed biofilms.

V. Co-cultivation of *C. glabrata* and *C. albicans* with *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) results in growth inhibition and attenuated virulence of these pathogenic yeasts

V.1. Abstract

In the previous chapters of this thesis, it had been described different aspects on the way by which *Candida* species may interact with indigenous lactobacilli of the vaginal flora and, in particular, with *L. gasseri*. In this chapter it was examined such interaction but with the intestinal species *L. reuteri* considering that both *C. albicans* and *C. glabrata* are also frequently found in the gut. The results obtained show that in co-cultures with *Candida*, *L. reuteri*, like *L. gasseri*, reduces viability and growth rate of the two yeasts while in planktonic growth; forms mixed biofilms involving close cell-cell contacts and inhibit the ability of *C. albicans* to undergo filamentation and to cause virulence against the wax *Galleria mellonella*. Despite this, prominent differences in the way by which *L. reuteri* and *Candida* interact under the tested conditions were found, compared to *L. gasseri* including: i) in the co-cultures with *L. reuteri* it was not observed the alkalinization of the broth prompted by *Candida* cells that counteracted the acidification caused by the accumulation of lactic acid in the medium; ii) the heterofermentative metabolism of *L. reuteri* results in the accumulation of acetic acid in the medium that is believed to cause additional toxic effects for the *Candida* cells, alone or in combination with lactic acid and eventually other metabolites produced by the bacteria. These findings indicate the existence of important species-specific effects that can determine the way by which *Lactobacillus* species interact with *Candida* and should be further scrutinized in order to foster what can be probiotic based treatments for invasive candidiasis progressing from the gut.

V.2. Introduction

Various *Lactobacillus* species are commonly found in the human gut such as *L. acidophilus, L. fermentum*, *L. rhamnosus* and *L. reuteri,* the species that is focused in this study[150, 151, 667]. Although in the past *L. reuteri* was classified as a *Lactobacillus* species, in a recent reorganization of the genus this species was reclassified and renamed as *Limosilactobacillus reuteri.* The great diversity among *Lactobacillus* species prompted the scientific community into reconsidering their classification based on old and new parameters that included core genome phylogeny, pairwise average amino acid identity, clade-specific signatures, physiological criteria and the ecology of the organisms[668]. For example, this reclassification divided homofermentative from heterofermentative lactobacilli and, as stated, reclassified *L. reuteri* in the *Limosilactobacillus* group which comprises species able to produce exopolysaccharides from sucrose ("slimy *Lactobacillus*")[668].

L. reuteri is considered an endogenous gut symbiont and has evolved suitable adaptation mechanisms to thrive in this host that assure colonization such as the expression of mucus-binding proteins (MUBs) that can bind to the intestinal epithelia, promoting adhesion[150, 240, 241]. The genome of the reference *L. reuteri* strain ATCC 23272 has approximately 1.99 Mb in size with a total predicted protein count of 1900 and a GC content of ~38.9% (data obtained from NCBI: Bioproject PRJNA15766). Unlike vaginal lactobacilli species, *L. reuteri* ferments glucose in a heterofermentative manner[224], meaning that it produces both lactic and acetic acid. While in the vaginal tract the most abundant lactobacilli are exclusively homofermentative (producing two molecules of lactic acid from glucose), in the gut there are both homo- (e.g. *L. ruminis* and *L. salivarius*) and heterofermentative lactobacilli[150, 151, 667]. As in the vaginal tract, most lactobacilli are widely regarded as biomarkers of health of the human gut due to their association with a healthy environment and this has been justifying the investment in research put in their study and, eventually, in their exploration as probiotics[134, 669-671]. Indeed, several studies have been showing good results in exploring *L. reuteri* in adults, children and infants as a probiotic treatment[382, 672-674] with the benefits including improved feed tolerance; enhanced absorption of nutrients, minerals, and vitamins; stimulation of host immune responses; enhanced gut mucosal integrity; and reduced bacterial translocations which consequently results in reduced frequency of infections originating from the gut[675-677]. Importantly, lactobacilli have been explored as anti-*Candida* therapies in the gut. Specifically, supplementation of neonates with *L. rhamnosus* (also found in the gut) resulted in a significantly reduction of *Candida* spp. colonization of the gut from 48.8% in the control groups to 23.1% in the probiotic group[381]. Additionally, infant supplementation with a probiotic containing *L. reuteri* was shown to be as effective as nystatin in reducing *Candida* colonization of the gut (18.7% compared with 16%) and skin (14% compared with 12%)[382] and a clinical trial of *in vivo* supplementation of newborns with *L. reuteri* showed that this species was a potent antagonist of *Candida* spp., effective in preventing the enteric colonization of the yeasts (reduced gut *Candida* load)[678].

In this work we analyse, in detail, the interaction established between *L. reuteri* and the pathogenic yeasts *C. albicans* and *C. glabrata*. Previous studies have shown that *L. reuteri* cell-free supernatants obtained from *L. reuteri* cultures inhibit growth of these two *Candida* species[679]. However, the use of bacterial supernatants does not recapitulate the aspects that could be related with the direct cell-cell interaction or aspects that could be specifically induced in the bacteria when *Candida* cells are present. For example, in chapter II, we showed that the pH dynamics observed along a co-culture established between *L. gasseri* and *Candida* species, cannot be recapitulated when only bacterial supernatants are used. As a consequence, a confounding effect is observed resulting from the presence of high concentrations of acetic acid, that are supplied in the MRS medium in the form of sodium acetate. Thus, it is of relevance the study of how these two species interact by exploring co-cultures. *L. reuteri* cells were also found to be able to co-aggregate with *Candida* cells resulting in inhibition of their growth[236, 680, 681].

Not much is known concerning the factors underlying these inhibitory effects caused by *L. reuteri* and this is also an objective of this study. This species was previously reported to produce considerable amount of biosurfactant[233, 234] and of exopolysaccharides[682, 683], these being some features that have been suggested to underlie the antimicrobial potential of *L. reuteri*, but their effect in specifically inhibiting *Candida* has not been fully addressed. *L. reuteri* is also known to produce the bacteriocin reutericin[229, 230], although to our best knowledge its specific effects against *Candida* have not been studied. *L. reuteri* is also able to produce the antimicrobial compounds reuterin (3-hydroxypropionaldehyde (3-HPA))[225, 226] and reutericyclin[227, 228], and reuterin was shown to inhibit both growth and biofilm formation of *C. albicans*[225, 226, 684]. *L. reuteri* has been also reported to produce phenyllactic acid[239], 2-hydroxyisocaproic acid[404] and 1-acetyl-β-carboline[395]. Phenyllactic acid was shown to impair biofilm formation of *C. albicans* and prolong the survival rate of *Galleria mellonella* larvae infected with this species[685], while 2-hydroxyisocaproic acid exerted a fungicidal effect against *C. albicans* and *C. glabrata*[403]. 1-acetyl-β-carboline was shown to block yeast-to-hypha transition in *C. albicans*, but has not able to cause inhibition in growth of these species[395].

V.3. Material and Methods

V.3.1. Strains and growth media

In this chapter were used the following strains: *Limosilactobacillus reuteri* ATCC 23272 (formerly *Lactobacillus reuteri*) (acquired from DSMZ), *Lactobacillus gasseri* ATCC 33323 (acquired from DSMZ), *C. glabrata* KUE100 (a wild-type strain derived from the *C. glabrata* reference strain CBS138[433]) and *Candida albicans* SC5314. The MRS medium used to co-cultivate yeasts and bacteria, contains, per litre, 10 g casein peptone (Gibco); 10 g meat extract (Panreac AppliChem); 5 g yeast extract (Gibco); 20 g glucose (Nzytech); 1 g Tween 80 (Sigma); 2 g K_2HPO_4 (Merck); 5 g sodium acetate (Merck); 3 g ammonium sulphate (Panreac AppliChem); 0.20 g $MgSO_4.7H_2O$ (Labchem) and 0.05 g $MnSO_4.H_2O$ (Sigma). After preparation, the pH of MRS was adjusted to 6.2-6.5 using HCl or NaOH. In indicated experiments, the sodium acetate used to prepare MRS was replaced by sodium chloride (Honeywell, Fluka™) or was added 3-morpholinopropane-1-sulfonic acid buffer (MOPS) to buffer the medium to a pH 6.5. YPD medium, used for maintenance of the yeast strains, contains, per litre, 20 g glucose (Nzytech), 20 g peptone (Gibco) and 10 g yeast extract (Gibco). Solid YPD or MRS were prepared by supplementing the corresponding liquid medium with 2% and 1.5% agar (Nzytech), respectively. Media were prepared using deionized water and sterilized by autoclaving for 15 min at 121°C and 1 atm.

V.3.2. Co-cultivation of *L. reuteri* with *C. glabrata* or *C. albicans*

To examine growth of *L. reuteri* in liquid MRS, alone or in the presence of *C. glabrata* or *C. albicans*, a pre-inoculum of each individual species was prepared in this medium and the cells cultivated, overnight, at 37ºC with an orbital agitation of 100 rpm. On the next day, these cells were used to inoculate (at an OD_{600nm} of 0.4 for *L. reuteri* and 0.1 for the two *Candida* species) fresh MRS medium. Growth in this co-culture system was accompanied for 4 days, at 37ºC and using an orbital agitation of 100 rpm, by following the cellular viability of the two species based on the number of colony-forming units (CFUs). For this, aliquots of co-cultures were taken, serially diluted, and plated on MRS supplemented with 96 mg/L fluconazole (an antifungal concentration that fully prevented the growth of *Candida* colonies and thus only *L. reuteri* colonies were visible) or in YPD supplemented with 300 mg/L tetracycline (an antibiotic concentration that fully prevented the growth of *L. reuteri* colonies and therefore only *Candida* colonies were visible). The number of *Candida* colonies formed onto the surface of YPD plates was counted after 2 days of incubation at 30ºC, while the number of *L. reuteri* colonies formed onto the surface of MRS plates was counted after 2 days of plate incubation at 37ºC in a Genbox (Biomerieux) with a candle inside to assure microaerophilia[541, 542]. As controls, single-cultivations of *L. reuteri*, *C. albicans* and *C. glabrata* in the same conditions used for the co-cultivations were performed. Quantification of the amounts of lactic acid, acetic acid or glucose present in the broth during single or multi-

species cultivation was performed by HPLC (equipped with an UV detector, for quantification of lactic and acetic acids, and with a RI detector, for quantification of glucose) using an Aminex HPX87H (Biorad®) column and 0.005M H_2SO_4 (at a flow rate of 0.6 mL/min of) as eluent. The same experimental setup was used to co-cultivated *C. glabrata*/*C. albicans* and *L. reuteri* in MRS having sodium chloride as a sodium source (instead of the usually used sodium acetate) or in MRS buffered to pH 6.5 using 3-morpholinopropane-1-sulfonic acid buffer (MOPS).

V.3.3. Co-cultivation of *L. reuteri* with *C. glabrata* or *C. albicans* under biofilm-forming conditions

To examine growth under biofilm-forming conditions of *L. reuteri*, alone or in co-cultivation with *C. albicans* or *C. glabrata*, a pre-inoculum of each species was prepared in MRS (or in this same medium containing sodium chloride as a sodium source or MOPS) and the cells were cultivated, overnight, at 37ºC, using an orbital agitation of 100 rpm. These pre-cultures were used to inoculate 200 µL of fresh MRS in plastic µ-slide 8 well plates (Ibidi) so that the initial cell densities (estimated based on OD_{600nm}) were 10^6 CFU/mL for the two *Candida* species and 10^8 CFU/mL for *L. reuteri*. After 24h of cultivation at 37ºC with 25 rpm agitation, the supernatant of the single or co-cultures was removed and the biofilm formed was washed with 200 µL of PBS. To assess cellular viability in the single- or multi-species biofilms formed, 3 µM of SYTO 9 Green Fluorescent Nucleic Acid Stain (Molecular Probes, Eugene, USA) was added to the single or co-cultures and the cells were left in the dark for 30 minutes. After this time, 4 µM TO-PRO-3 iodide (Molecular Probes, Eugene, OR, USA) was added and the cultures were incubated under the same conditions for another 15 minutes. The gain adjustment in each channel was optimized (and kept during the experiments) taking into account the intensity fluorescence signal of live and dead single cells. Live single cells were stained directly after growth, while dead single cells were prepared by heating a cell sample at 65ºC for 10 minutes in a dry bath. Then, single or multiple species biofilms were imaged by confocal laser scanning microscopy using a Leica TCS SP5 inverted microscope with a 63x water (1.2 numerical aperture) apochromatic objective. Cells were imaged with the 488 nm Ar^+ laser line to detect cells stained with SYTO 9 (emission collected at 500 – 590 nm) and with the 633 nm He-Ne laser line to detect cells stained with TO-PRO-3-Iodide (emission collected at 645-795 nm), a setup that minimizes cross-interference between the two channels as described in Pinto *et al.*, 2019[543]. Scanning electron microscopy (SEM) was performed similarly to the aforementioned protocol but biofilms were prepared in 2.5 mL MRS in 5 mL polystyrene plates. After 24h of incubation at 37ºC with 25 rpm agitation, the supernatant was removed and the cells adhered to the surface of the plate were dehydrated using the following protocol: washing with distilled water; washing with 70% ethanol for 10 minutes; washing with 95% ethanol for 10 minutes; and washing with 100% ethanol for 20 minutes. Plates were then dried in a desiccator for at least 96h and visualized by scanning electron microscope (SEM) using a magnification between 5000x and 10000x with a high voltage of 15.0 kV.

V.3.4. Effect of *L. reuteri* or *L. gasseri* in filamentation undertaken by *C. albicans*

Yeast-to-hyphae transition prompted by *C. albicans* was assessed by co-cultivation of this species together with *L. reuteri* and *L. gasseri* in RPMI, at pH 7 and at pH 4. For this, mid-exponential phase cells of *C. albicans* (obtained from a pre-culture performed also in RPMI) were inoculated in RPMI with *L. reuteri* or *L. gasseri* cells (obtained from a pre-culture performed in MRS medium) in a proportion of 1:4 yeast:bacteria (corresponding to OD_{600nm} of 0.1 of the yeasts and of 0.4 of the bacteria). To induce filamentation, 10% FBS (fetal bovine serum) was further added to the medium. The cultures were incubated for 24h, at 37ºC and 100 rpm, after which they were imaged in an optical Zeiss microscope equipped with a 1000x magnification lens. The assay was repeated but using inactivated lactobacilli cells, obtained by incubating the bacteria pre-culture sample to be inoculated in the RPMI medium at 65ºC, for 10 minutes in a dry bath.

V.3.5. Effect of *L. reuteri* on virulence of *C. glabrata* against the infection model wax *Galleria mellonella*

To assess how the presence of *L. reuteri* affected the virulence of *C. albicans* against the infection model wax *Galleria mellonella* an experimental methodology previously explored in the lab[686] was used. Briefly, a microsyringe was used to inject 5 µL of a suspension of *Candida* cells (containing approximately 10^9 CFU/mL) in the last left proleg of each caterpillar. Following injection, larvae were placed in glass Petri dishes and stored in the dark at 37 °C. For each condition, at least 10 larvae were tested, being followed their survival over a period of 72 hours. Caterpillars were considered dead when they displayed no movement in response to touch. To assess the effect of *L. reuteri*, approximately 10^5 CFUs of this bacterium were added to the food used to feed the larvae and when these were considered mature were inoculated with *C. glabrata* following the methodology described above.

V.4. Results

V.4.1. Planktonic co-cultivation of *C. glabrata* and *C. albicans* with *L. reuteri* results in reduced growth and viability of the yeasts

We started this study by analysing the impact of the direct co-cultivation of *C. albicans* or *C. glabrata* with *L. reuteri*, in similar terms to those before with the vaginal species *L. gasseri*. As such, we have examined how the co-cultivation setting impacted growth parameters and viability of the involved species. As in Chapter II, in which these same aspects were investigated for *L. gasseri*, we have examined the co-cultures both in planktonic and in biofilm-forming conditions. For the planktonic cultures, we started using ~100 more bacterial than yeast cells resulting in a total amount of ~10^8 CFUs/mL *L. reuteri*, compared to 10^6 CFUs/mL of *C. albicans* or *C. glabrata* (Figure V.1).

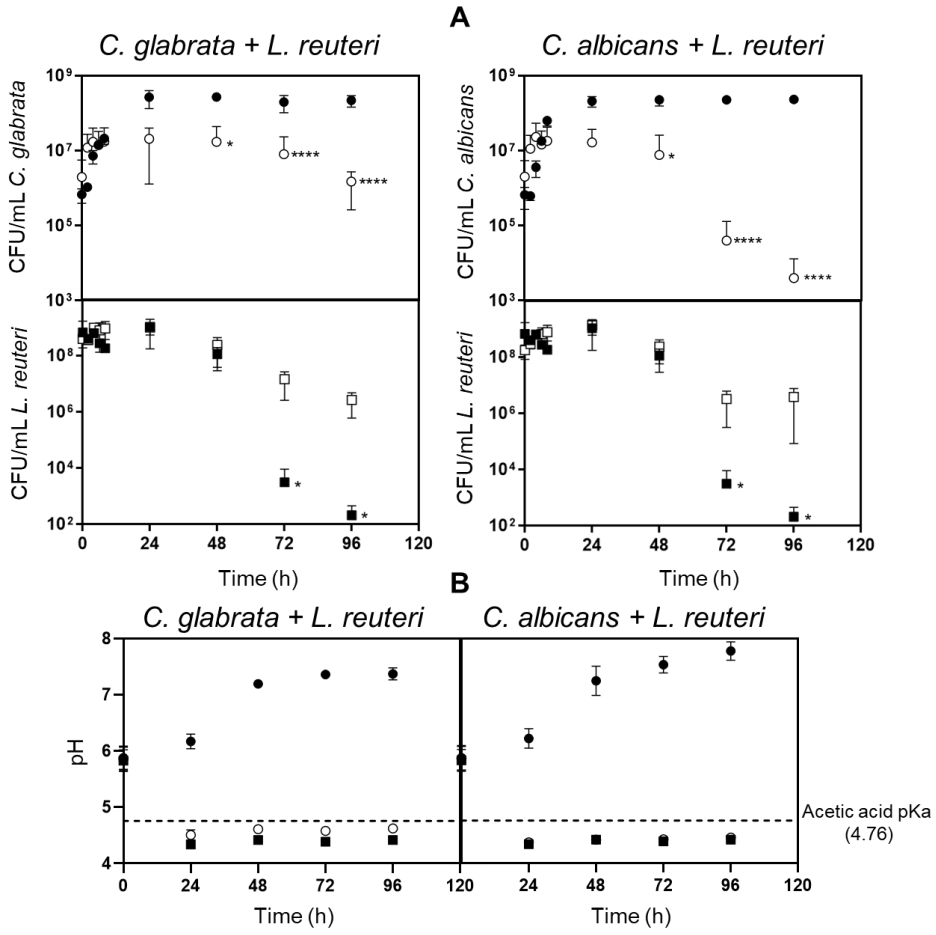


Figure V.1. (A) Cellular viability of *C. glabrata*, *C. albicans* and *L. reuteri* along single or co-cultivation in MRS medium. After inoculation, cells of *C. albicans* (○,●), *C. glabrata* (○,●) or *L. reuteri* (□,■) were cultivated at 37ºC and 100 rpm for 96h with the growth of the different species being accompanied based on cellular viability, as detailed in materials and methods. Filled symbols correspond to the samples taken during single-species cultivation while open symbols correspond to the samples taken during co-cultivation. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001). **(B)** Variation of medium pH during cultivation of *L. reuteri* (■), *C. glabrata* or *C. albicans* (●) in MRS alone or in co-cultivation (○) under the same conditions as those used to obtain the growth curves shown in panel A.

Similarly to what was observed in Chapter II for *L. gasseri*, the viability of *L. reuteri* population while in single-culture decreased along time, but this was considerably attenuated when these cells were co-cultivated with the two *Candida* spp. (Figure V.1 panel A). While in single culture the decrease in *L. gasseri* viability was attributed to the accumulation in MRS medium of undissociated acetic acid (resulting from the protonation of the sodium acetate supplied as a source of sodium) that exerts powerful toxic effect in the cells. Co-cultivation with the two *Candida* species abrogated this effect since both of them were capable of alkalinizing the broth to values above acetic acid pKa, thus assuring that the predominant form was acetate (and not acetic acid). In the single-cultures of *L. reuteri* production of lactic acid to levels close to 5 g/L was observed (Annex Figure V.1) resulting in a final pH of 4.4, maintained constant at this value along the time took to complete the experiment. It is important to denote that by being heterofermentative, *L. reuteri* also produced acetate increasing the total amount to around 100 mM (Annex Figure V.1). In this context, it is likely that the observed loss of cell viability observed in the single-culture *L. reuteri* population could result from this exceeding accumulation of acetic acid. Under the experimental conditions used for the co-cultivation (100 rpm of agitation and 37ºC) the yeast cells resumed growth immediately after re-inoculation and maintained it until 24h, after which they entered stationary phase (Figure V.1 panel A). The bacterial population also increased, although much less prominently than the yeasts, likely due to an already higher starting population, comparing with the one of *Candida* (Figure V.1 panel A). Reflecting the more competitive and challenging environment of the co-culture, the growth rates of *C. glabrata* and *C. albicans* decreased 61% and 63%, respectively, comparing to those obtained in single-culture. Specifically, the growth rate of *C. glabrata* decreased from 0.25 h^{-1} in single-culture to 0.1 h^{-1} in co-culture, while the growth rate of *C. albicans* decreased from 0.24 h^{-1} in single-culture to 0.09 h^{-1} in co-culture (Figure V.1 panel A). Co-cultivation with *L. reuteri* resulted in a significant reduction in cellular viability of the two yeasts (of about 93% 99%) after 48 hours, with the effect being more pronounced for *C. albicans* than *C. glabrata* and increasing along time (as shown in Figure V.1 panel A). In this co-culture, *L. reuteri* produced ~5 g/L lactic acid when co-cultivated with *C. albicans* and 2.5 g/L when co-cultivated with *C. glabrata* (Annex Figure V.1). In the two co-cultures it was also denoted the production of ~40 mM acetate, bringing the total amount of acetate present to 100 mM (considering the initial 60 mM present in the medium). Concomitant with the production of lactic and acetic acids, the pH in the two co-cultures decreased rapidly and was maintained in the range of 4.3 and 4.5 for *C. albicans* and *C. glabrata*, respectively (Figure V.1 panel B). In this case it was not evident the alkalinization of the fermentation broth that was observed in the co-cultures undertaken with *L. gasseri* and, consequently, it is impossible to distinguish whether the reduced viability of the yeasts (and of the bacteria as well) results from the toxic effects of the accumulation of undissociated acetic acid or from the presence of *L. reuteri*, or from a combination of the two.

To clarify this matter, the co-cultivation was repeated but this time using MRS without any sodium acetate (sodium was supplied as sodium chloride). The results obtained, shown in Figure V.2, reveal a powerful acceleration of viability loss in *L. reuteri* cells while in single-culture, that

was not observed when the cells were cultivated in the presence of the yeasts (Figure V.2 panel A).

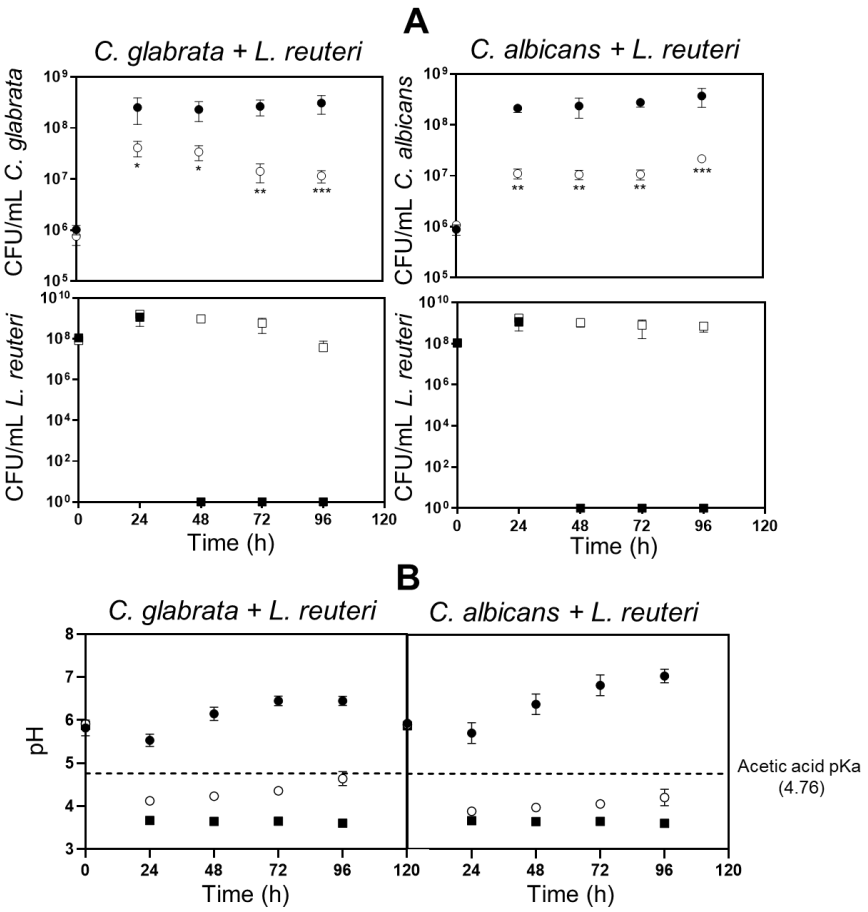


Figure V.2. (A) Cellular viability of *C. glabrata*, *C. albicans* and *L. reuteri* along single or co-cultivation in MRS medium without sodium acetate. After inoculation, cells of *C. albicans* (○,●), *C. glabrata* (○,●) or *L. reuteri* (□,■) were cultivated at 37ºC and 100 rpm for 96h with the growth of the different species being accompanied based on cellular viability, as detailed in materials and methods. Filled symbols correspond to the samples taken during single-species cultivation while open symbols correspond to the samples taken during co-cultivation. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001). **(B)** Variation of medium pH during cultivation of *L. reuteri* (■), *C. glabrata* or *C. albicans* (●) in MRS-NaCl alone or in co-cultivation (○) under the same conditions as those used to obtain the growth curves shown in panel A.

Expectedly, under these conditions *L. reuteri* also produced acetate, but the final amount (~20 mM) was now well below the one observed in standard MRS. Notably, the pH of the single-cultures undertaken in MRS-NaCl achieved 3.6 (compared to 4.3 that was obtained using standardized MRS medium), which is attributable to a buffering effect caused by sodium acetate. In the co-cultures with *C. glabrata* and *C. albicans* were visible two effects: i) growth of the yeasts appeared to be more limited, compared with the one observed in single-cultures, achieving ODs at stationary phase that were smaller (this effect was particularly visible for *C. albicans*; ii) viability of *C. glabrata* in the co-culture was reduced along time (Figure V.2). A decrease in pH was observed also in these two co-cultures, concomitant with the production of lactic acid (2.5 g/L in the co-cultivation with *C. glabrata* and 5 g/L in co-cultivation with *C. albicans* (Annex Figure V.2)) and acetic acid (achieving 50mM in the co-cultivation with *C. glabrata* and 40mM in co-cultivation

with *C. albicans* (Annex Figure V.2)), to a value close to 4 (Figure V.2 panel B). Under these conditions it is also conceivable that the observed decreased viability of the yeasts (or their limited growth) results from the acetic acid accumulated in the undissociated form (estimated to be in the range of 12-20 mM for the time-point of 96h taking into account the final pH and the amount of acetate quantified in the broth), however, in this case this acetic acid is produced by the bacteria and not part of the medium. It is also important to note that previous studies undertaken to assess tolerance levels of *C. glabrata* to acetic acid stress show almost no growth inhibition at this range of concentrations and pH[430].

To determine whether the capability of *L. reuteri* to inhibit growth of *Candida* goes beyond the production of acetic acid, we repeated the co-cultivations in standardized MRS medium (that is the one containing 60mM sodium acetate) but buffering it to pH 6.5 using 3-morpholinopropane-1-sulfonic acid buffer (MOPS) (Figure V.3).

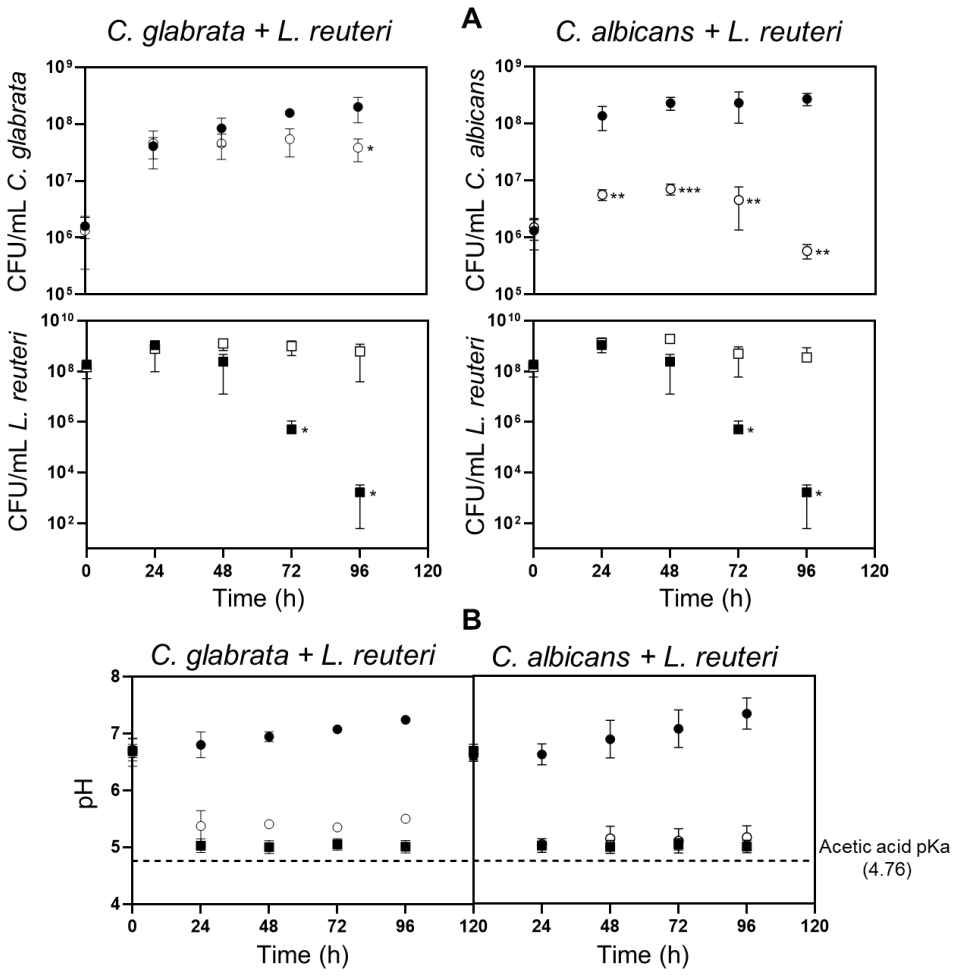


Figure V.3. (A) Cellular viability of *C. glabrata*, *C. albicans* and *L. reuteri* along single or co-cultivation in MRS medium buffered with MOPS. After inoculation, cells of *C. albicans* (○,●), *C. glabrata* (○,●) or *L. reuteri* (□,■) were cultivated at 37ºC and 100 rpm for 96h with the growth of the different species being accompanied based on cellular viability, as detailed in materials and methods. Filled symbols correspond to the samples taken during single-species cultivation while open symbols correspond to the samples taken during co-cultivation. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001). **(B)** Variation of medium pH during cultivation of *L. reuteri* (■), *C. glabrata* or *C. albicans* (●) in MRS (buffered to pH 6.5 using MOPS) alone or in co-cultivation (○) under the same conditions as those used to obtain the growth curves shown in panel A.

Under these conditions a reduction in viability of *L. reuteri* cells while in single-cultures was observed, similarly to what had been observed in the other two settings and suggesting that this effect is not only attributable to the accumulation of acetic acid (since in this case the pH was maintained at 5 along the experiment, thus being above the acid pKa (Figure V.3 panel B)). Remarkably, viability of the bacterial cells in the co-cultivation is maintained suggesting that the yeasts are, somehow, sustaining bacterial viability. In the co-cultures it was still visible the reduction in viability of the yeast population, especially in the case of *C. albicans* were this was considerably more pronounced (Figure V.3 panel A). Despite the production of acetic acid is also observed in these two co-cultivations, the pH was maintained at 5 and thus there is no accumulation of toxic undissociated acetic acid and we attribute the loss of yeast cell viability to the presence of the bacteria.

V.4.2. While in co-cultivation in sessile conditions, *C. glabrata*/*C. albicans* form mixed biofilms with *L. reuteri*

To study the formation of mixed biofilms between the two *Candida* species and *L. reuteri*, the same experimental setting used for *L. gasseri* was utilized, with the objective of promoting the formation of biofilms in 8-well microplates that were, afterwards, imaged by confocal microscopy and small scanning microscopy (SEM). Under the conditions detailed in materials and methods, after 24h of cultivation in MRS, both *C. albicans* and *C. glabrata* formed a mixed biofilm with *L. reuteri* as shown by the scanning electron microscopy (SEM) images depicted in Figure V.4. Noteworthy, in some regions of the mixed biofilm formed between *L. reuteri* and *C. glabrata* it was observed what appear to be a thick extracellular matrix that was not found in other regions of the plate and it was also not found in the mixed biofilms involving *C. albicans* (Figure V.4 and Annex Figure V.3). To understand the outcome for the viability of the different species of the formation of these mixed biofilms, the same fluorescence microscopy assay with SYTO9 and TO-PRO-3 iodide labelling used before was utilized (shown in Figure V.5). The results obtained showed significant red labelling in the biofilms formed only by *L. reuteri*, however, closer looking into the image revealed that this labelling was not confined to the cells but actually correspond to what appeared to be an extracellular matrix (insert in Figure V.5). Since TO-PRO-3 iodide binds to DNA, it is possible that this labelling corresponds to the binding of the dye to nucleic acids accumulated in the extracellular matrix of the biofilm (Figure V.5). The same observation was also made in the biofilms formed by *L. gasseri*. However, unlike the mixed biofilms formed between *Candida* and *L. gasseri* that did not exhibited this “extracellular labelling”, in the mixed biofilms formed with *L. reuteri* such labelling was observed (Figure V.5). It was also noticeable that the number of yeast cells present in these mixed biofilms was considerably reduced, compared to those formed with *L. gasseri*, suggesting a predominance of the bacteria over the *Candida* cells that prevent them to form the biofilms (Figure V.5). It was also evident from the imaging of the mixed biofilms several *L. reuteri* red-labelled cells (note the increased number of red-labelled cells

in the central image of Figure V.5) indicating that some loss of bacterial viability occurs which was also not observed for *L. gasseri*.

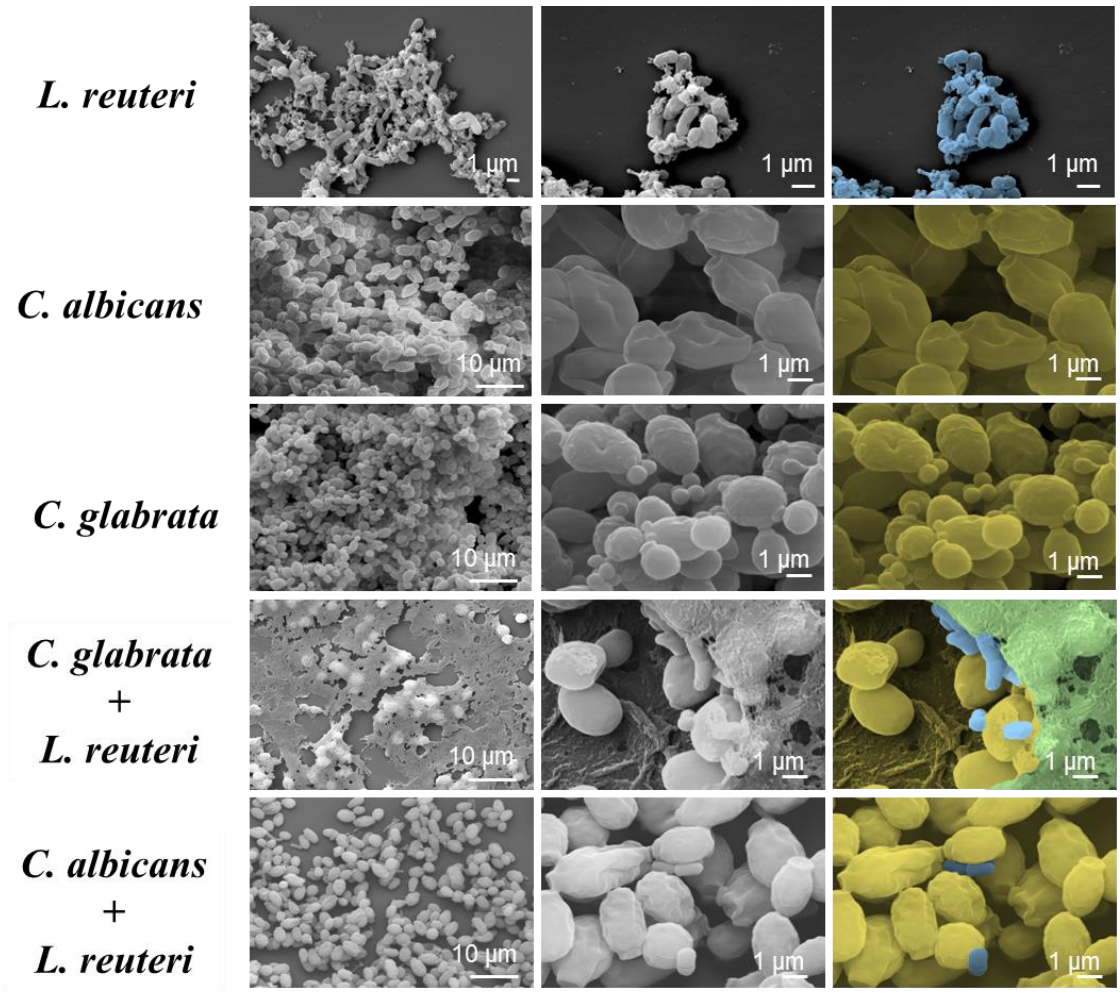


Figure V.4. Biofilms formed by *L. reuteri*, *C. albicans* and *C. glabrata* during single or co-cultivation in MRS, as detected by scanning electron microscopy. Note that *L. reuteri* and *C. glabrata* mixed biofilms contain what appears to be an extracellular matrix (green). The biofilms were obtained using the same conditions described in materials and methods, with the exception that was used 5 mL-polystyrene plates instead of the 8mm plates used in the fluorescence microscopy imaging. These images were obtained after 24h of cultivation at 37ºC and 25 rpm using a magnification of 5000x to 10000x over the dried biofilms. For the sake of facilitating the identification of cells, in the last column, the yeast cells were labelled in yellow, while the bacterial cells were labelled in blue.

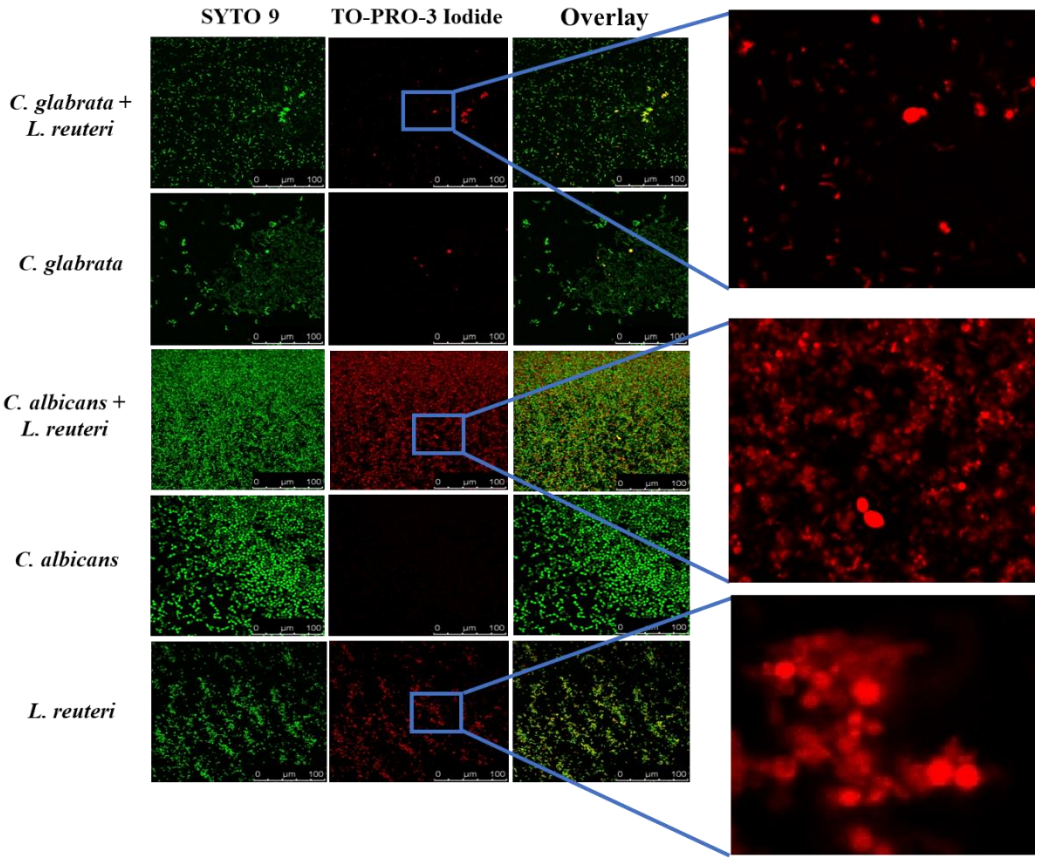


Figure V.5. Live/dead imaging of cells in single-species or in mixed biofilms formed by *L. reuteri*, *C. albicans* or *C. glabrata* after 24h of cultivation, at 37ºC and 25 rpm, in MRS. The images presented are representative of a set taken from the biofilms in three replica experiments performed. *L. reuteri* and *Candida* spp. single and mixed biofilms scale bar corresponds to 100 µm.

Since we could not directly link the results obtained with the TO-PRO-3 iodide fluorescence directly with cellular viability (this would require an individualized quantification of fluorescence in bacterial and in yeast cells), these experiments were repeated (by the student Joana Valério that kindly allowed me to use her results in this thesis) under the same conditions but instead of labelling the mixed biofilms, we used a methodology to tear them apart after which aliquots were plated in different solid media to assess the number of CFUs/mL of each species (Figure V.6).

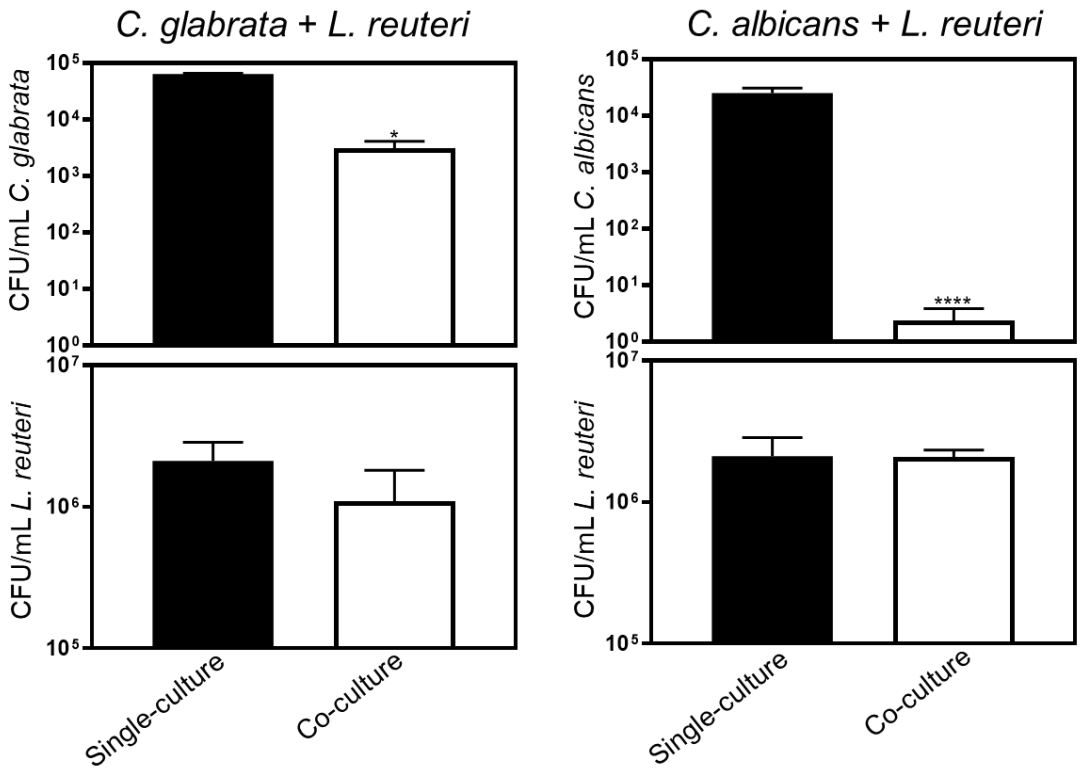


Figure V.6. Cellular viability of single-species or mixed biofilms formed by *L. reuteri*, *C. albicans* or *C. glabrata* after 24h of cultivation, at 37ºC and 25 rpm, in MRS containing 60mM sodium acetate. The viability of the different species was determined using the same plating method as for the planktonic growth, as detailed in materials and methods. The CFUs presented were obtained from three independent replicates. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

Using this approach we could confirm some loss of viability of *L. reuteri* cells in the mixed biofilms (more evident in those formed with *C. glabrata*), in line with the observations made in the imaging made with confocal microscopy. We could also observe that the CFU/mL of *Candida* cells was lower, decreasing almost completely for *C. albicans* and of about 20-fold for *C. glabrata*, comparing to the levels that could be determined in the biofilms formed in the absence of the bacteria.

V.4.3. Effect of *L. reuteri* on virulence traits of *C. glabrata* and *C. albicans*

Taking into account the potential of *L. reuteri* to inhibit growth of *Candida* it was decided to examine whether these bacterial cells would also have the capability of restraining virulence of these yeasts, especially in a co-culture setting that has not yet been investigated. We started by examining the effect of co-cultivation in the yeast-to-hyphae transition in *C. albicans* since this is crucial for full pathogenesis of this species[268, 687]. We started by trying to assess this in MRS medium, the same that was used above to co-cultivate *L. reuteri* and *Candida* cells, however, under these conditions we could not detect any significant filamentation of the yeast cells (results not shown). This incapacity to filament could be linked with the highly complex composition of the MRS medium that could inhibit filamentation, but also with the pH of the broth that reached values

near 4 after 24h of co-cultivation (it is documented that low pH reduces *C. albicans* filamentation[688]), or possibly from a synergy between these two factors. Thus, we repeated the co-cultivation in RPMI at pH 7 supplemented with FBS since this is described to be a potent filamentation inducer[689]. Under these conditions, it was possible to denote a clear reduction in the number of filamenting *C. albicans* cells in the co-culture with *L. reuteri*, a phenotype partly rescued when the incubation was made with inactivated bacterial cells (Figure V.7). Co-incubation with the vaginal species *L. gasseri* under these conditions also resulted in decreased number of *C. albicans* filamenting cells, albeit in this case the difference was milder than the one observed with *L. reuteri* (Figure V.7). The same observations were made when performing this assay in RPMI pH 4 (Annex Figure V.4). Altogether, it appears that filamentation of *C. albicans* was highly affected by both lactobacilli and that this inhibition occurred both in acidic and neutral pHs.

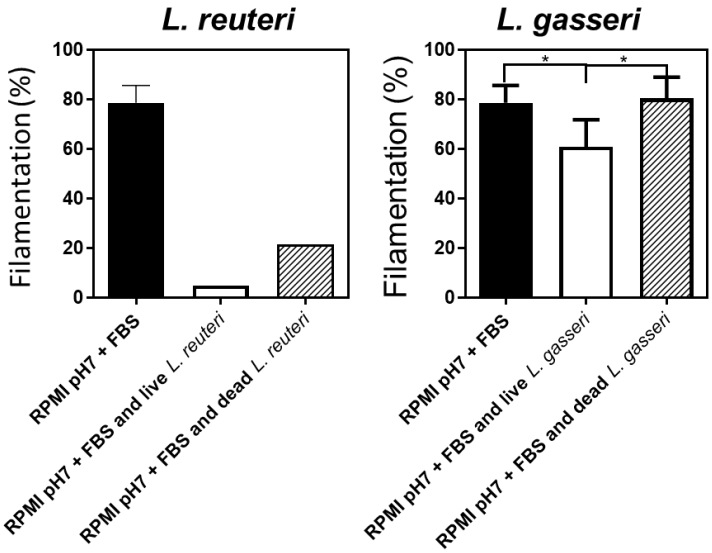


Figure V.7. Filamentation ratio of *C. albicans* in the presence of *L. reuteri* and *L. gasseri* live and dead cells. Growth was performed at 37ºC at 100 rpm for 24h in RPMI pH 7 supplemented with 10% FBS to induce filamentation. This data was obtained from a large set of images taken using an optical microscope with a magnification of 1000x. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001). *L. gasseri* data was obtained from three different replicates, while *L. reuteri* was only obtained from one replicate.

We have also examined how virulence of *Candida* against the infection model *Galleria mellonella* could be modulated by the presence of *L. reuteri*. In a first approach we tried to do a co-infection directly in the blood lymphae, however all injected larvae died, presumably due to the very high microbial burden. We took thus a different approach and supplied *G. mellonella* food with *L. reuteri* so that maturation of the larvae could occur in the presence of the bacteria, after which we would then inject the larvae with *C. glabrata*. Under this setup, we could show that feeding *G. mellonella* with *L. reuteri* supplemented food during larvae growth reduced the mortality rate prompted by *C. glabrata* by about 50% after 48 hours of yeast injection (Figure V.8).

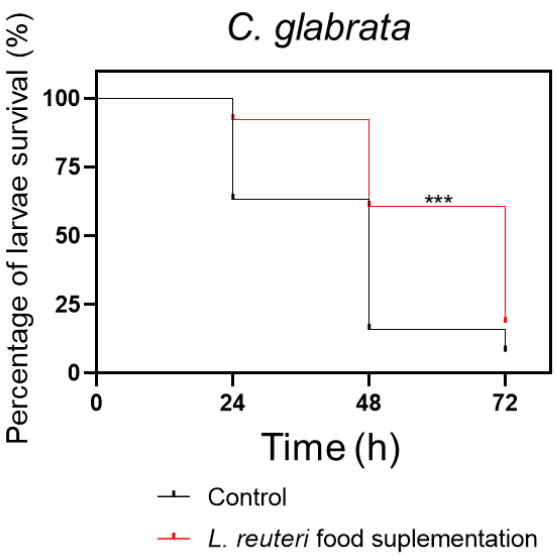


Figure V.8. *L. reuteri* reduces the virulence traits of *C. glabrata*. Survival rate of *G. mellonella* after infection with *C. glabrata* in larvae previously fed with *L. reuteri* supplemented food. The mortality rate of *G. mellonella* was monitored for a period of 72h. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

V.5. Discussion

This chapter focused on the interaction established between the endogenous intestinal species *L. reuteri* and the pathogenic yeasts *C. albicans* and *C. glabrata*. *L. reuteri* has been extensively used in probiotics, with several reports demonstrating that improves gut health through the production of antimicrobial molecules responsible for inhibiting the colonization of pathogenic microbes, by modulating host immune system through the reduction of pro-inflammatory cytokines and by strengthening the intestinal barrier[239, 677, 690, 691]. Our results demonstrated that co-cultivation of *L. reuteri* with *C. glabrata* and *C. albicans* in MRS medium reduces both the growth rate and the cellular viability of the yeasts, while also resulting in improved viability of the bacteria. Notably, this phenotype was also obtained when we made the co-cultivations in MRS having NaCl as the sodium source and also in MOPS-buffered MRS. This means that the *Candida* cells exert a beneficial effect in augmenting viability of the *L. reuteri*, a trait that was also observed in the co-cultivation established between these same two *Candida* species and *L. gasseri*. However, in the case of the co-cultures with *L. gasseri*, this beneficial effect was attributed to the alkalinization of the broth prompted by the yeast cells and that avoided the accumulation in the MRS medium of toxic concentrations of acetic acid. In this case, in the co-cultures established between *L. reuteri* and *C. glabrata* such buffering effect was not observed, neither in MRS, neither in MRS-NaCl and the final pH of the co-cultures was always in the range of 4.4-4.5. This creates an important effect caused by the accumulation in the broth of undissociated acetic acid molecules (whose pKa is 4.76) that have a described antimicrobial effect and can thus underlie the reductions observed in viability observed both for the yeast and for the bacterial cells. This accumulation of undissociated acetic acid results from the acetate already provided in the standardized MRS medium, but also from acetate that is naturally produced along fermentation of glucose by *L. reuteri*. Besides this mechanism that involves the production of acetic acid, other mechanisms appear also to underlie the reduction in viability of *Candida* while growing in the presence of *L. reuteri* since a reduction in viability of these cells was observed even when the co-cultivation was made in MOPS-buffered medium at pH 5 (above acetic acid pKa). *L. reuteri* produces other metabolites described to affect *Candida* cells out of which reuterin, an intermediate of glycerol catabolism to 1,3-propanediol[231, 232]. Under the conditions that we used for HPLC analysis of the fermentation broth of the single and co-cultures we could not detect production of propanediol, however, further studies will be required to demonstrate if indeed there is no production and if the analytical conditions used were adequate to make such measurement.

The fact that along the co-cultivation of *Candida* with *L. reuteri* the yeasts were unable to alkalinize the growth medium as they do when co-cultivated with *L. gasseri* was quite intriguing. Although it could be hypothesized that the higher concentration of acetate accumulating in the broth of *L. reuteri* (because of its heterofermentative metabolism) could result in a higher buffering capacity of the medium, inhibiting eventual alkalinization mechanisms prompted by *Candida*, we do not favour this hypothesis since *Candida* cells were able to increase pH in co-cultures with *L.*

gasseri in standard MRS that also contained approximately 50mM acetic acid, the same amount that was present in the co-cultures with *L. reuteri* in NaCl-MRS (Annex Figure II.4 panel A and Annex Figure V.2). Further studies will be required to investigate if, for example, *L. reuteri* produces a compound that inhibits the excretion of the alkalinizing molecule(s) that *Candida* cells are using to increase pH while in co-culture with *L. gasseri*.

We also investigated an eventual role of *L. reuteri* in inhibiting biofilm formation prompted by *C. glabrata* and *C. albicans*. In this case we used the same experimental design used for *L. gasseri* (in Chapter II) resorting to confocal fluorescence microscopy. As previously stated, this method enables to distinguish less viable cells among bacterial and fungal cells. In mixed biofilms, we observed that the presence of *L. reuteri* reduces the numbers of *Candida* cells, more prominently of *C. albicans*. However, unlike what was performed with *L. gasseri*, in this case it was not possible to quantify this loss of viability because *L. reuteri* cells (and possibly its extracellular matrix) were also stained with TO-PRO-3 iodide. Directly determining the number of CFUs/mL in the biofilm, we could demonstrate that *L. reuteri* viability is not significantly affected by the presence of the yeasts, while *C. glabrata* and *C. albicans* viability was severely inhibited. This was in line with other reports showing impact of *L. reuteri* presence in biofilm formed by *C. albicans*[692].

A hypothesis currently under study is the fact that acetate has previously been demonstrated to be an inducer of bacteriocin production in other lactobacilli[564, 565] and required for the anti-*Candida* potential of *L. gasseri*[605]. While *L. gasseri* lost most of its anti-*Candida* potential in MRS without acetate (Chapter II), when co-cultivated with the *Candida* spp. *L. reuteri* retained its anti-*Candida* potential. A crucial difference is the capacity of producing acetic acid of these species. *L. reuteri* can produce considerably large amounts of acetic acid compared with *L. gasseri*[239, 605]. The fact that *L. reuteri* produced a significant amount of acetic acid makes it impossible to determine the outcome of removing the acetate from the growth medium since it is naturally produced by this species to the point that it accumulates in the broth. Inevitable, this could result in the inhibition of *Candida* spp. directly by the produced acetic acid or activation of an anti-*Candida* potential modulated by it similar to what was observed for *L. gasseri*[605], or even both.

We have also demonstrated herein that *L. reuteri* and *L. gasseri* cells reduces filamentation prompted by *C. albicans*, and even more importantly, these cells were more effective in reducing filamentation when these cells were metabolically active (the effect is reduced when the lactobacilli cells are killed). It is possible that viable cells are more effective in reducing filamentation through the production of secondary metabolites. One of such metabolites could be 1-acetyl-β-carboline, shown to be produced by *L. reuteri* strains and to have a strong anti-filamentation potential against *C. albicans*[395]. Further metabolomic studies will be required to investigate which other molecules could also underlie this effect of *L. reuteri* and eventually its capacity to induce loss of cell viability in *Candida*. Another relevant virulence trait of *Candida* spp is to induce a systemic infection in the host. For this was used the infection model organism *G.*

mellonella. Feeding these larvae with *L. reuteri* during their growth before infection with *C. glabrata* resulted in the reduction of the mortality prompted by the yeast. Previous results have demonstrated that several species of *Lactobacillus* (e.g., *L. paracasei* and *L. rhamnosus*), were able to modulate the immune system of *G. mellonella* and protect against *Candida* infection[693, 694], and thus it is possible that *L. reuteri* could be exerting its protective effect by modulating the innate immune system of *G. mellonella* and not directly inhibiting the proliferation of the *Candida* cells. A future prospect would be to evaluate the impact of *L. reuteri* in the expression of *G. mellonella* innate immune system related genes.

VI. Final discussion

The surging of new *Candida* spp. resistance to the commonly used antifungal therapies is highly problematic, especially considering the high rates of morbidity and mortality associated with systemic infections and the recurrence and persistence of superficial infections that are accompanied by an overall reduction of quality of life and increased health burden for society. The current shortage of antifungal alternatives in the market increases the interest in developing new anti-*Candida* therapies based on other active principles and targets. It is also expected that this effort can foster the identification of novel therapeutic targets and also identify new molecules that can be used alone, or in combination with classical antifungals, to control the emergence of resistant strains. This thesis research focused on the hypothesis of using live probiotics to treat *Candida* infections. The human host microbiota has been correlated with the overall state of health in various human host niches, including the vagina and the gut. Among the organisms that comprise the human microbiota, *Lactobacillus* are considered pivotal for the well-being and health maintenance of the human host niches. An example is the vaginal microbiota, where lower lactobacilli abundance was correlated with the increased risk of bacterial vaginosis[132, 133, 695, 696]. However, the same correlation between lactobacilli abundance and the development of *Candida* infections is still unclear[102, 136-139]. Nonetheless, lactobacilli supplementation has been correlated with the reduced *Candida* load and attenuation of *Candida* infections symptoms in the vaginal and intestinal tract[372, 377, 381, 382]. Additionally, *in vitro* reports have demonstrated that lactobacilli are potent antagonists of *Candida* growth and virulence traits[327, 429, 435, 578, 582]. However, most of these studies focus on *Lactobacillus* species not considered endogenous to the human host, mostly because of our poor understanding of these autochthonous species, despite the observations that they are crucial to maintain a healthy environment. Altogether, these evidences support the hypothesis that probiotics, including the ones formulated with endogenous lactobacilli species, could be used to treat and prevent *Candida* infections. This thesis focused specifically on the use of endogenous lactobacilli species of the human host (vaginal and intestinal), and their potential to be used as probiotics against *Candida* spp. To be precise, it focused on the vaginal species *L. gasseri* and the intestinal species *L. reuteri*.

In this thesis we strongly invested in the study of the direct co-cultivations of *Candida* and *Lactobacillus*, instead of what had been made for most works where the anti-*Candida* potential was determined using lactobacilli supernatant as a proxy[389, 390, 397]. This effort paid-off because we could demonstrate in the case of *L. gasseri*, that its single-culture supernatants do not phenocopy what happens in the co-cultivation settings. In particular *L. gasseri* single-culture supernatants in MRS were very acidic and thus contained high concentration of acetic acid (supplied in the form of sodium acetate to the medium) creating a confounding effect (is the inhibition caused by the lactobacilli or by the accumulation of acetic acid?) while in the co-cultivation *Candida* cells could alkalinize the medium above the pKa of the acid and thus we do not observe the accumulation of acetic acid (Chapter II). On the other hand, using *L. reuteri* we

could not completely avoid this issue because the *Candida* spp. were not able to alkalinize the broth. This confounding effect was even more significative because of its heterofermentative metabolism that resulted in the accumulation of acetic acid concentrations in the supernatant, in contrast with *L. gasseri* that has a homofermentative metabolism. Still, we could demonstrate that there is a potential anti-*Candida* effect of both lactobacilli. In *L. gasseri* we could observed a decrease of yeasts viability after the alkalinization (inhibition is not caused by the acetic acid) of the medium while in the case of *L. reuteri* we could demonstrate an inhibition of the yeasts in conditions buffered above the pKa of the acetic acid. Thus, we consider the main outcomes of this thesis the observations that *L. gasseri* and *L. reuteri* can antagonize both *Candida* spp. and that studying a co-culture setting is not the same as studying the bacterial supernatant.

One of the main objectives of this thesis was to identify competitiveness genes of *C. glabrata* and *L. gasseri*, information that could be leveraged to develop better probiotic strains (obtained from the identification of *L. gasseri* competitiveness genes) or new anti-*Candida* therapies targeting *C. glabrata* competitiveness genes that could tip the ecological balance in favour of the healthy endogenous bacteria. For this we used two high throughput analyses based on transcriptomics and phenomics resulting in the model shown in Figure VI.1. Our first approach of using transcriptomics was used with the intention of unveiling, more from an academic point of view, the molecular mechanisms responsible for *C. glabrata* and *L. gasseri* interaction. Note that, this knowledge is crucial for the identification of competitiveness genes in this species that could be used to develop new anti-*Candida* therapies. Importantly, differentially expressed genes are not necessarily crucial for competitiveness. Thus, our second approach, consisted of using phenomics on a *C. glabrata* mutant collection with single deletion strains. We focused on the identification of genes that would be responsible for *Candida* growth in the presence of *L. gasseri*. Unfortunately, we could only search for competitiveness genes in *C. glabrata* during the time frame of this thesis because of the poor knowledge regarding *L. gasseri* molecular tools and the lack of a mutant collection in this species. In Figure VI.1 we describe the main molecular findings of *C. glabrata* and *L. gasseri* responsible for adaptation to co-cultivation and the genes of *C. glabrata*, that from phenotypic observations appear to be responsible for competitiveness of *C. glabrata* against *L. gasseri* (regardless of the growth being performed in planktonic or sessile conditions or the data obtained from transcriptomics or phenomics approaches). From both these approaches we could determine that only, *CAGL0L03828g*, *CAGL0F04499g*, *CAGL0L10208g*, *EPA1*, *CAGL0D00220g* and *DIG1* were up-regulated and required for competitiveness. This small number of genes could result from the fact that up-regulated genes could not be required for competitiveness or result from the small number of genes tested from the mutant collection that comprises only 578 *C. glabrata* mutants strains, a number considerably low compared with the total number of genes in this species. In fact, from the 425 genes up-regulated after 8h of growth only 25 were present in the *C. glabrata* mutant collection and phenotyped for competitiveness against *L. gasseri*, which hardness the task of corroborating our transcriptomics results regarding the identification of true competitiveness genes. Interestingly, *CAGL0D00220g* and *DIG1* were found to be up-regulated in co-cultivation settings (planktonic growth) but only required for

competitiveness in biofilm forming conditions. It is important to mention that the identified *C. glabrata* competitiveness genes in the planktonic versus the biofilm conditions were distinct, possibly reflecting what is a very different growth environment. Nonetheless we cannot exclude that the phenomics analysis in planktonic conditions was performed in pooled growth using barcode sequencing of the mutants in a pool that contained many *C. glabrata* mutant strains simultaneously, thus this method could be less sensitive compared with the approach used for the biofilm screening (fluorescence spectroscopy) where the mutants were grown separately. Differences between these pooled (planktonic) and single mutant (biofilm) growth methodologies can be related with the fact that the pooled assay has greater number of variables that could influence its outcome including barcode amplification efficiency, sequencing bias or simply the mutants being less fit to growth in pooled conditions even in the absence of *L. gasseri*.

As previously mentioned, in Chapter III and Chapter IV we focused on the identification of genes of *C. glabrata* and *L. gasseri* responsible for adaptation to co-cultivation and the *C. glabrata* genes required for competitiveness against *L. gasseri*, respectively and their biological function. Many of the genes from *C. glabrata* and *L. gasseri* that were differentially expressed were assigned to the COG classes involved with translational, ribosomal structure and biogenesis, transcription, amino acid metabolism and carbohydrate transport and metabolism. This being in line with previous reports focusing on the co-cultivation of *L. rhamnosus* and *L. crispatus* with *C. albicans*[140, 398]. *C. glabrata* carbohydrate and transport metabolism included the up-regulation of *HXT1* and down-regulation of *HXT5* and *HXT4/6/7* after 8h of co-cultivation, suggesting a different affinity for glucose of transporters. Additionally, many of its genes related to carbon metabolism were down-regulated (Table III.3). In *L. gasseri* we observed a strong up-regulation of the phosphotransferase (PTS) sugar transport system[697, 698], including *LGAS_RS02465*, *LGAS_RS00730* and *LGAS_RS08210* responsible for carbon uptake. While we could observe a differential expression of many genes belonging to this biological function, mostly down-regulated, we could not identify competitiveness genes related to carbon transport and metabolism in *C. glabrata* apart from the glucose responsive transcription factor *RGT1* (not differentially expressed), which regulates the expression of several glucose transporter (HXT) genes in response to glucose[625]. Thus, suggesting that in co-cultivation, *C. glabrata* cells can rewire their metabolism in face of less optimal growth conditions (less carbon availability for example) without any impact on their ability to grow in the presence of *L. gasseri*. This was expected since cells have different genes with redundant functions that compensate for the single gene deletion of the mutant strain. Regarding competition for amino acids, and consequently nitrogen, *C. glabrata* showed simultaneous down and up-regulation of genes related to amino acid metabolism and uptake while *L. gasseri* was practically characterized by an up-regulation of these genes (*LGAS_RS02795*, *LGAS_RS08890*, *LGAS_RS04265*, *LGAS_RS00240*). Interestingly, the genes *ARG8* and *ARG1*, involved in arginine metabolism were up-regulated (not present in the mutant collection thus were not phenotyped) in *C. glabrata* and *ARG7* was found to be required for competitiveness against *L. gasseri* although it was not differentially expressed. Altogether these observations point to a role of arginine biosynthesis on the survival of *C. glabrata* in co-cultivation.

We could also observe an up-regulation of several oxidative stress response genes of *C. glabrata* (e.g., *TSA1*, *CTA1* and *TRR2*) while *SKN7* transcription factor, which is a core regulator of the oxidative stress response of *C. glabrata*[649, 699] was not differentially expressed. Although *SKN7* was not differently expressed, it was found to be required for competitiveness against *L. gasseri*. The fact that *SKN7* was not differentially expressed suggests that *L. gasseri* activates this factor through other mechanisms (e.g., protein oxidation and phosphorylation[700]) and not through modulation of its genomic expression. In addition to the *TSA1*, *CTA1* and *TRR2* genes, transcriptomics revealed that four other genes (acireductone dioxygenease (*ADI1*), cytochrome c peroxidase (*CCP1*), *SCM4* (ORF uncharacterized) and cystathionine beta-lyase (*STR3*)), regulated by *SKN7*, were also up-regulated at the earliest growth stage (2 hours). Additionally, we determined that, although *C. glabrata* Hog1 protein was previously reported to be required for growth in the presence of lactobacilli, including *L. gasseri*[635], it was down-regulated at 2 hours of co-cultivation. However, phenotypic analysis revealed that deletion of the upstream genes of the HOG signalling pathway (*MSB2*, *HKR1*, *PBS2* and *SHO1*) resulted in the increased susceptibility of *C. glabrata* to *L. gasseri*. Hog1 protein is known to transduce its signal through a cascade of protein phosphorylation[641]. We found evidence that disrupting this cascade either by deleting Hog1 protein, as previously reported[635] or by deleting the upstream elements *MSB2*, *HKR1*, *PBS2* and *SHO1*, responsible for signal transduction, results in the competitiveness decrease of *C. glabrata* to the presence of *L. gasseri*. *C. glabrata* Hog1 protein was shown to be required for survival when challenged with physiological concentrations of lactic acid[635]. This suggests that the HOG signalling pathway in *C. glabrata* takes a role on competitiveness against *L. gasseri*, possibly by mediating survival in a high lactic acid concentration environment, that could be taken into consideration as a potential therapeutic target that could tip the ecological balance towards lactobacilli. Additionally, the transcriptional regulator *CRZ1* and an upstream gene of the calcineurin pathway, *MID1*, were found to be down-regulated in co-cultivations with *L. gasseri* in the early growth stage. Both these genes, *CNA1* and *CCH1* (not differentially expressed) were found to be essential for growth in the presence of *L. gasseri*. As explained in Chapter IV these genes belong to the calcineurin signalling pathway which is responsible for various biological functions in the cells, including antifungal susceptibility, virulence, thermotolerance, biofilm formation and pH homeostasis in *C. glabrata*[45, 46]. Among the up-regulated genes of *C. glabrata* and simultaneously regulated by *CRZ1*, only *YPS5*, a member of the glycosylphosphatidylinositol-linked aspartyl proteases shown to be required for virulence in mice[290] was found. Although *CRZ1* is a transcriptional regulator protein, and thus their activity could be attributed to other protein modifications besides its genomic expression, *MID1* is a calcium transporter that when deleted resulted in decreased *C. glabrata* competitiveness and thus should be up-regulated in co-cultivation settings to counteract the deleterious effects of *L. gasseri*. The fact that *CRZ1* and *MID1* were down-regulated after 2 hours of growth but required for competitiveness could be explained by the fact that this pathway can be less required in the early growth stage but become increasingly relevant in later co-cultivation period. This aspect is meaningful because transcriptomics analysis was performed after 2h and 8h of co-cultivation

while the phenomics analysis was performed after 24 hours of growth, which results in a higher accumulation of *L. gasseri* metabolites in the medium and consequently affects other mechanisms that could have escaped from our transcriptomic analysis. Nonetheless, although this pathway remains to be scrutinized for its role in protecting *C. glabrata* against *L. gasseri*, it appears that it may be a good candidate as a target for future anti-*Candida* therapies. For all these pathways, although we could provide evidence that they are important for competitiveness, due to the lower number of deletion strains tested, it remains to be identified the downstream effectors responsible for *C. glabrata* competitiveness to *L. gasseri*, this being an important line of study to be explored in the future.

To summarize, in this thesis we could identify a wide number of genes responsible for adaptation in co-cultivation conditions belonging to various biological processes including ribosomal encoding genes, carbon and transport metabolism, osmotic and oxidative stress, adhesins, lactate metabolism and MDR transporters in *C. glabrata* while in *L. gasseri* we identified a set of genes related with carbon PTS transport and amino acid transport (detailed in the model represented in Figure III.9). Unfortunately, we could not overlap most of these genes with the ones obtained from the phenomics analysis. However, we could further add to our model the roles of the HOG and calcineurin signalling pathways (detailed in the model Figure IV.13). Overall, this thesis was successful in exploring and unveiling the anti-*Candida* potential of human lactobacilli, mostly focused on the interaction between *C. glabrata* and *L. gasseri*. Specifically, we could determine that both *L. gasseri* and *L. reuteri* are able to inhibit the pathogenic yeasts *C. glabrata* and *C. albicans* growth and virulence traits. Importantly, this anti-*Candida* potential appears to be modulated by the presence of acetate, at least in *L. gasseri*, which allowed us to add to our final model the role of the acetate, although it remains unclear how it modulates the expression of possible anti-*Candida* agents such as bacteriocins, which production has already been linked with the presence of acetate in the medium[565, 583-585]. Altogether, these findings were in line with this thesis goal of gathering information on *Candida*/*Lactobacillus* interaction that could be used to develop better probiotics strains using endogenous lactobacilli species and develop new anti-*Candida* therapies that could tip the ecological balance towards lactobacilli.

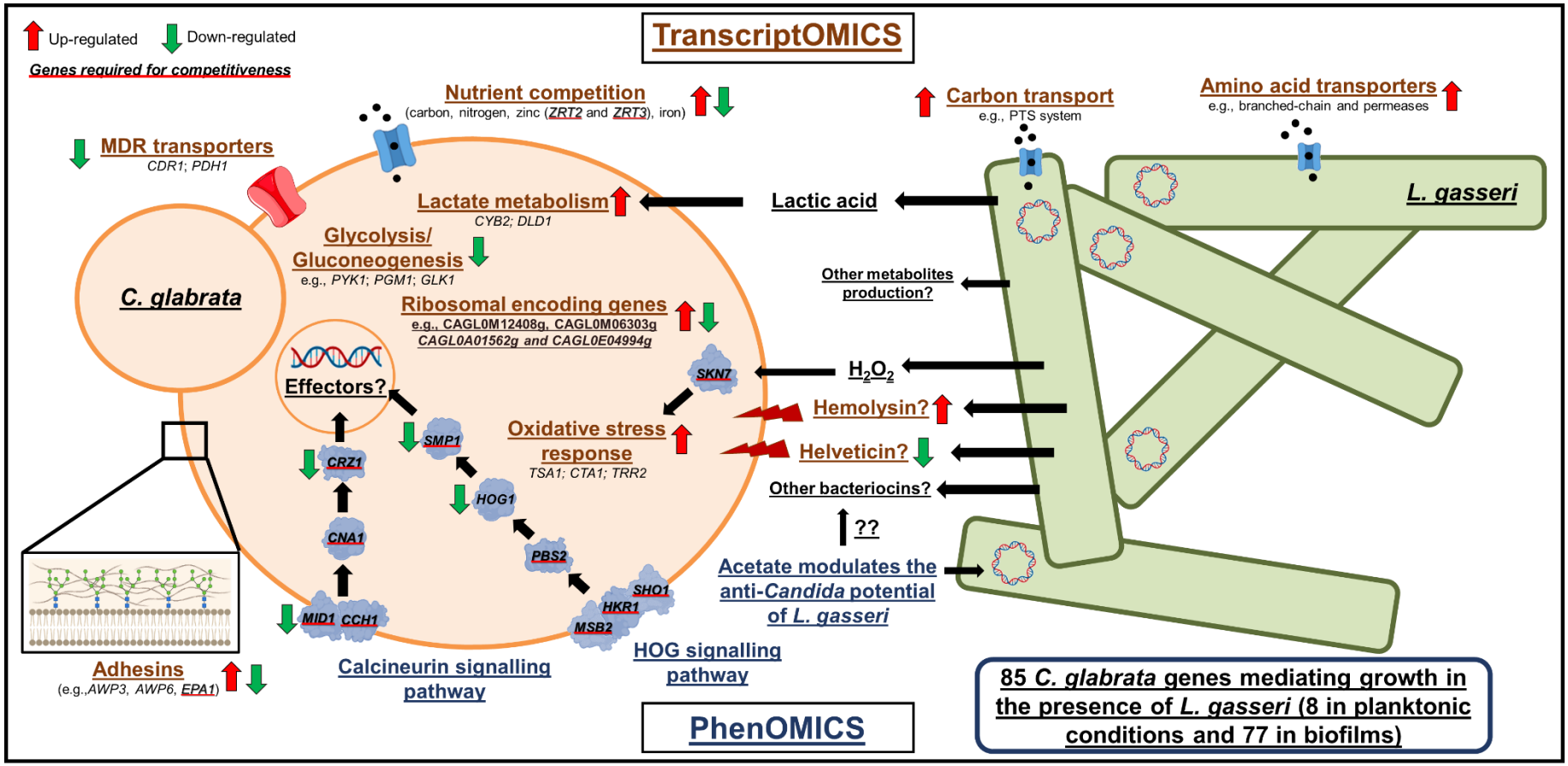


Figure VI.1. Model representing the genomic players modulating *C. glabrata* and *L. gasseri* interaction including genes responsible for competitiveness in co-cultivation settings. Genes of *C. glabrata* phenotyped and required for competitiveness in the presence of *L. gasseri* are underlined in red. Red arrows indicate the up-regulation while the green arrows indicate the down-regulation of most genes associated with each biological function. Note that some biological classes have simultaneously up and down-regulated genes. This model was built using the information gathered with the high throughput methods of transcriptomics (in orange) and phenomics (in blue) and is intended to summarize the models described in Figure III.9 and Figure IV.13.

Another outcome of this thesis was an update of the hypothesized anti-*Candida* mechanisms of lactobacilli responsible for their probiotic properties that was shown in the chapter I of this thesis. In addition to the previously identified potential anti-*Candida* agents (e.g., biosurfactants, exopolysaccharides, bacteriocins, H_2O_2 and 1-acetyl-β-carboline) we were able to further investigate how lactobacilli exert their anti-*Candida* properties, mostly focused on the vaginal tract. Thus, we provide in Figure VI.2, an updated view of the possible mechanisms of lactobacilli responsible for *Candida* inhibition in the vaginal tract. Historically, lactic acid has been considered a potential anti-*Candida* agent, however, from work in the laboratory we determined that under physiologically relevant conditions lactic acid is not likely the major anti-*Candida* agent[430]. This would be expected taking into consideration the pH and pKa of lactic acid, resulting in a lower concentration of the undissociated lactic acid in the vaginal tract (around 30% of the total lactic acid), the fact that *Candida* spp. have evolved mechanisms that allow for immune system evasion in the presence of lactic acid[289] and due to their ability to consume lactate as a sole carbon source[433, 434]. All these arguments point to the conclusion that lactic acid is not responsible for the inhibition of *Candida* in the vaginal tract. However, we attribute to lactic acid the ability to reduce the medium pH, consequently affecting the undissociated/dissociated ratios of other weak acids. Phenyllactic acid and 2-hydroxyisocaproic, produced by lactobacilli, have been previously demonstrated to be effective antifungals agent[398-400, 402-404], and with a pKa of 4.02 and 4.26 respectively, these have a higher ratio of undissociated/dissociated, compared with lactic acid, and thus could be potential lactobacilli agents against *Candida in vivo* in low pH environments. Although this model works for the vaginal tract, which is an acidic environment, in the gut the pH is not expected to contribute to the anti-*Candida* potential of lactobacilli since it is a very alkaline niche (~5 to 7)[431]. From this work we have also identified the potential role of hemolysins in inhibiting *C. glabrata*, although this subject remains to be further pursued, and have concluded that *L. gasseri* reduced the growth, filamentation and biofilm formation of *Candida*. Additionally, during this thesis, we have identified acetate as a potential modulator of the anti-*Candida* properties of *L. gasseri*. Although it remains to be identified the mechanisms affected by the presence of acetate in *L. gasseri*, it was previously reported that the presence of acetate could modulate bacteriocin production in other gram-positive bacteria, including *L. plantarum*, *L. sakei*, and *L. rhamnosus*[565, 583-585], exopolysaccharides in *L. reuteri*[701] and H_2O_2 in other gram-positive bacteria (e.g., *Weissella cibaria*)[702] which are compounds that could have a role in contributing for the ecological balance of *Candida*/*Lactobacillus* in the vagina (Figure VI.2).

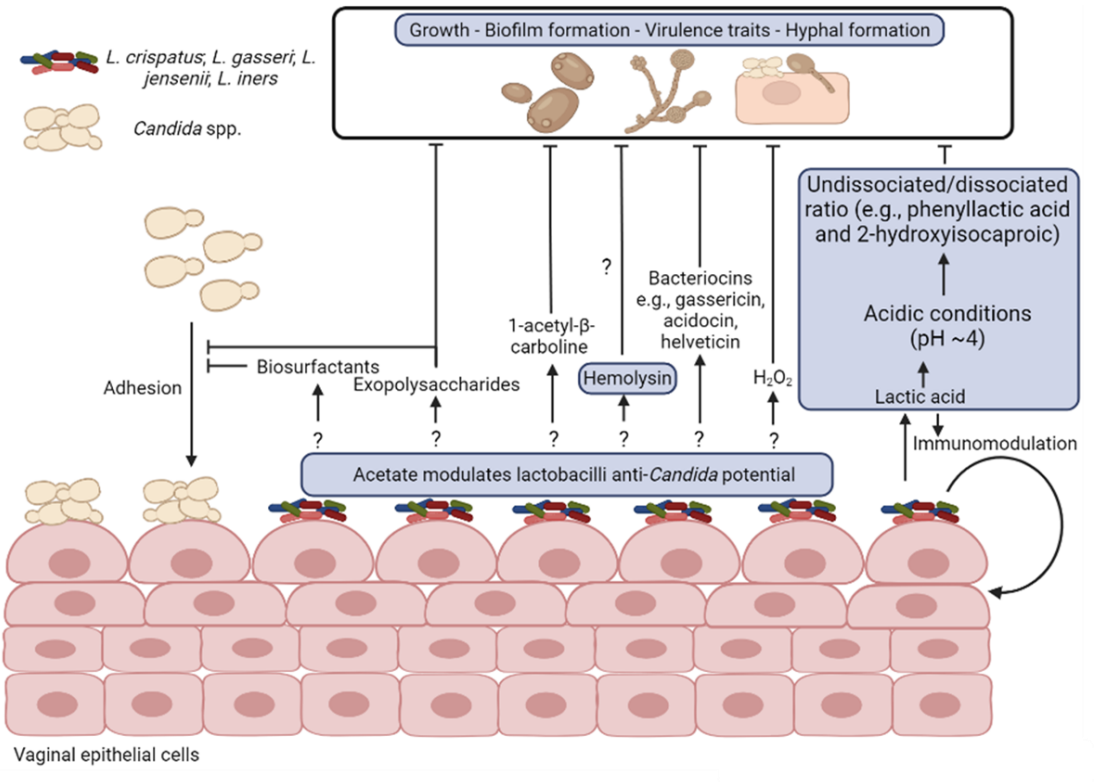


Figure VI.2. Proposed anti-*Candida* mechanisms of lactobacilli. Lactobacilli production of biosurfactants, exopolysaccharides, H_2O_2, bacteriocin, 1-acetyl-β-carboline and lactic acid (indirectly) could reduce *Candida* colonization of the vaginal tract. The novel findings of this work include (in the blue squares): the observation that the indirect effect of lactic acid could be responsible for the changes in the undissociated/dissociated of other weak acids with anti-*Candida* properties (e.g., phenyllactic acid and 2-hydroxyisocaproic); the potential role of hemolysin in inhibiting *Candida* spp; and the identification of the role of acetate in inducing the anti-*Candida* mechanisms of *L. gasseri*.

VII. List of Publications and Communications

The following outputs were obtained from the research described in this thesis, either directly (stemming from results herein shown) or indirectly (from research works that are related with this thesis, although chosen not to be herein presented).

Peer-reviewed scientific publications:

Marques LM, Alves MM, Eugénio S, Salazar SB, **Pedro N**, Grenho L, Mira NP, Fernandes MH, Montemor MF. Potential anti-cancer and anti-*Candida* activity of Zn-derived foams. Journal of Materials Chemistry B. 2018;6(18):2821-30.

Lourenço A, **Pedro NA**, Salazar SB, Mira NP. Effect of acetic acid and lactic acid at low pH in growth and azole resistance of *Candida albicans* and *Candida glabrata*. Frontiers in microbiology. 2019 Jan 8;9:3265.

Gonçalves B, Bernardo R, Wang C, Schröder MS, **Pedro NA**, Butler G, Azeredo J, Henriques M, Mira NP, Silva S. Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: A transcriptomic analysis. International Journal of Medical Microbiology. 2020 Apr 1;310(3):151414.

Salazar SB, Simões RS, **Pedro NA**, Pinheiro MJ, Carvalho MF, Mira NP. An overview on conventional and non-conventional therapeutic approaches for the treatment of candidiasis and underlying resistance mechanisms in clinical strains. Journal of Fungi. 2020 Mar;6(1):23.

Pedro, N. A., Fontebasso, G., Pinto, S. N., Alves, M., & Mira, N. P. (2023). Acetate modulates the inhibitory effect of *Lactobacillus gasseri* against the pathogenic yeasts *Candida albicans* and *Candida glabrata*. *Microbial Cell*, *10*(4), 88.

Oral communication in national or international meetings:

Nuno A. Pedro and Nuno P. Mira. Study of the interaction between vaginal lactobacilli, *Candida albicans* and *Candida glabrata*: from physiological aspects to transcriptomic analyses. Presented to XXI Jornadas de Biologia de Leveduras "Professor Nicolau van Uden", Braga, Campus de Gualtar, University of Minho, 8-9, June (2018).

Nuno Pedro, Catarina Lima, Andreia Pimenta, Dalila Mil-Homens Sandra C Pinto, Marta Alves, Arsénio Fialho and Nuno Mira. Study of the interaction between vaginal and intestinal lactobacilli, *Candida albicans* and *Candida glabrata*. Presented to 15th International Congress on Yeasts (ICY), 23 -27 August (2021), Virtual meeting.

Nuno Pedro, Catarina Lima, Andreia Pimenta, Dalila Mil-Homens Sandra C Pinto, Marta Alves, Arsénio Fialho and Nuno Mira. Fostering the development of “live pharmaceuticals” based on probiotic lactobacilli for the treatment of candidiasis. Presented to 2nd iBB Workshop, 23 September (2022), Lisbon, Portugal.

Poster communication in national or international meetings:

Nuno A. Pedro, Marta Alves and Nuno P. Mira. Study of the interaction between vaginal lactobacilli, *Candida albicans* and *Candida glabrata*: from physiological aspects to transcriptomic analyses. Poster presented at 14th ASM Conference on *Candida* and Candidiasis, Providence, USA, 15-19, April (2018).

Nuno A Pedro, Catarina Lima, Andreia Pimenta, Sandra C Pinto, Marta Alves, Arsénio Fialho and Nuno P Mira. Study of the interaction between vaginal lactobacilli, *Candida albicans* and *Candida glabrata*: from physiological aspects to transcriptomic analyses. Poster presented at HFP2019: Human Fungal Pathogens: Molecular Mechanisms of Host-Pathogen Interactions and Virulence, Nice, France, 18-24, May (2019).

Nuno A Pedro, Catarina Lima, Andreia Pimenta, Dalila Mil-Homens, Sandra C Pinto, Marta Alves, Arsénio Fialho, Nuno Mira. Study of the interaction between vaginal and intestinal lactobacilli, *Candida albicans* and *Candida glabrata*. Poster presented at Microbiology Society Conference on *Candida* and Candidiasis 2021, 21-27 March (2021), Virtual meeting.

Nuno Pedro, Catarina Lima, Andreia Pimenta, Dalila Mil-Homens, Sandra C Pinto, Marta Alves, Arsénio Fialho, Nuno Mira. Study of the interaction between vaginal and intestinal lactobacilli, *Candida albicans* and *Candida glabrata*. Poster presented to Microbiotec21, 2021, 23-26 November, Nova University Lisbon (Virtual meeting).

Nuno Pedro, Carolina Glória, Catarina Lima, M Joana Pinheiro, Andreia Pimenta, Dalila Mil-Homens Sandra C Pinto, Marta Alves, Arsénio Fialho, Ana Azevedo, Nuno Mira. Towards the use of bio-based products as anti-*Candida* agents: from the use of lactobacilli species to bee venom. Poster submitted to the 36th International Specialized Symposium on Yeasts (ISSY36), 12-15 July, 2022 in Vancouver, Canada.

Awards:

Best poster award in Microbiotec21 on the subject “Microbiome in Health & Disease: microbial diversity and host-microbes interactions”, 2021, 23-26 November, Nova University Lisbon (Virtual meeting). Poster title: Study of the interaction between vaginal and intestinal lactobacilli, *Candida albicans* and *Candida glabrata*.

VIII. References

1. Berg G, Rybakova D, Fischer D, Cernava T, Verges MC, Charles T, Chen X, Cocolin L, Eversole K, Corral GH, Kazou M, Kinkel L, Lange L, Lima N, Loy A, Macklin JA, Maguin E, Mauchline T, McClure R, Mitter B, Ryan M, Sarand I, Smidt H, Schelkle B, Roume H, Kiran GS, Selvin J, Souza RSC, van Overbeek L, Singh BK, et al. (**2020**). Microbiome definition re-visited: old concepts and new challenges. **Microbiome** 8(1): 103. doi: 10.1186/s40168-020-00875-0.
2. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (**2007**). The human microbiome project. **Nature** 449(7164): 804-810. doi: 10.1038/nature06244.
3. Manor O, Dai CL, Kornilov SA, Smith B, Price ND, Lovejoy JC, Gibbons SM, Magis AT (**2020**). Health and disease markers correlate with gut microbiome composition across thousands of people. **Nat Commun** 11(1): 5206. doi: 10.1038/s41467-020-18871-1.
4. Maslowski KM, Mackay CR (**2011**). Diet, gut microbiota and immune responses. **Nat Immunol** 12(1): 5-9. doi: 10.1038/ni0111-5.
5. Zmora N, Suez J, Elinav E (**2019**). You are what you eat: diet, health and the gut microbiota. **Nat Rev Gastroenterol Hepatol** 16(1): 35-56. doi: 10.1038/s41575-018-0061-2.
6. Cuevas-Sierra A, Ramos-Lopez O, Riezu-Boj JI, Milagro FI, Martinez JA (**2019**). Diet, Gut Microbiota, and Obesity: Links with Host Genetics and Epigenetics and Potential Applications. **Adv Nutr** 10(suppl_1): S17-S30. doi: 10.1093/advances/nmy078.
7. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ (**2014**). Diet rapidly and reproducibly alters the human gut microbiome. **Nature** 505(7484): 559-563. doi: 10.1038/nature12820.
8. Modi SR, Collins JJ, Relman DA (**2014**). Antibiotics and the gut microbiota. **J Clin Invest** 124(10): 4212-4218. doi: 10.1172/JCI72333.
9. Benedict C, Vogel H, Jonas W, Woting A, Blaut M, Schurmann A, Cedernaes J (**2016**). Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. **Mol Metab** 5(12): 1175-1186. doi: 10.1016/j.molmet.2016.10.003.
10. Karl JP, Margolis LM, Madslien EH, Murphy NE, Castellani JW, Gundersen Y, Hoke AV, Levangie MW, Kumar R, Chakraborty N, Gautam A, Hammamieh R, Martini S, Montain SJ, Pasiakos SM (**2017**). Changes in intestinal microbiota composition and metabolism coincide with increased intestinal permeability in young adults under prolonged physiological stress. **Am J Physiol Gastrointest Liver Physiol** 312(6): G559-G571. doi: 10.1152/ajpgi.00066.2017.
11. Ying S, Zeng DN, Chi L, Tan Y, Galzote C, Cardona C, Lax S, Gilbert J, Quan ZX (**2015**). The Influence of Age and Gender on Skin-Associated Microbial Communities in Urban and Rural Human Populations. **PLoS One** 10(10): e0141842. doi: 10.1371/journal.pone.0141842.
12. Zozaya M, Ferris MJ, Siren JD, Lillis R, Myers L, Nsuami MJ, Eren AM, Brown J, Taylor CM, Martin DH (**2016**). Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. **Microbiome** 4(1): 1-10. doi: 10.1186/s40168-016-0161-6.
13. Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, Caporaso JG, Knights D, Clemente JC, Nakielny S (**2013**). Cohabiting family members share microbiota with one another and with their dogs. **elife** 2(e00458. doi: 10.7554/eLife.00458.
14. Cook MD, Allen JM, Pence BD, Wallig MA, Gaskins HR, White BA, Woods JA (**2016**). Exercise and gut immune function: evidence of alterations in colon immune cell homeostasis and microbiome characteristics with exercise training. **Immunol Cell Biol** 94(2): 158-163. doi: 10.1038/icb.2015.108.
15. Hou K, Wu ZX, Chen XY, Wang JQ, Zhang D, Xiao C, Zhu D, Koya JB, Wei L, Li J, Chen ZS (**2022**). Microbiota in health and diseases. **Signal Transduct Target Ther** 7(1): 135. doi: 10.1038/s41392-022-00974-4.
16. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, et al. (**2011**). Enterotypes of the human gut microbiome. **Nature** 473(7346): 174-180. doi: 10.1038/nature09944.
17. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, Program NCS, Bouffard GG, Blakesley RW, Murray PR, Green ED, Turner ML, Segre JA (**2009**). Topographical and temporal diversity of the human skin microbiome. **Science** 324(5931): 1190-1192. doi: 10.1126/science.1171700.

18. Mancabelli L, Milani C, Fontana F, Lugli GA, Tarracchini C, Turroni F, van Sinderen D, Ventura M (**2022**). Mapping bacterial diversity and metabolic functionality of the human respiratory tract microbiome. **J Oral Microbiol** 14(1): 2051336. doi: 10.1080/20002297.2022.2051336.
19. Price TK, Wolff B, Halverson T, Limeira R, Brubaker L, Dong Q, Mueller ER, Wolfe AJ (**2020**). Temporal Dynamics of the Adult Female Lower Urinary Tract Microbiota. **mBio** 11(2). doi: 10.1128/mBio.00475-20.
20. Aragon IM, Herrera-Imbroda B, Queipo-Ortuno MI, Castillo E, Del Moral JS, Gomez-Millan J, Yucel G, Lara MF (**2018**). The Urinary Tract Microbiome in Health and Disease. **Eur Urol Focus** 4(1): 128-138. doi: 10.1016/j.euf.2016.11.001.
21. Gajer P, Brotman RM, Bai G, Sakamoto J, Schutte UM, Zhong X, Koenig SS, Fu L, Ma ZS, Zhou X, Abdo Z, Forney LJ, Ravel J (**2012**). Temporal dynamics of the human vaginal microbiota. **Sci Transl Med** 4(132): 132ra152. doi: 10.1126/scitranslmed.3003605.
22. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ (**2011**). Vaginal microbiome of reproductive-age women. **Proc Natl Acad Sci U S A** 108 Suppl 1(Suppl 1): 4680-4687. doi: 10.1073/pnas.1002611107.
23. Ding T, Schloss PD (**2014**). Dynamics and associations of microbial community types across the human body. **Nature** 509(7500): 357-360. doi: 10.1038/nature13178.
24. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D (**2009**). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. **Clin Infect Dis** 49(4): 543-551. doi: 10.1086/600885.
25. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE (**2005**). Defining the normal bacterial flora of the oral cavity. **J Clin Microbiol** 43(11): 5721-5732. doi: 10.1128/JCM.43.11.5721-5732.2005.
26. Arweiler NB, Netuschil L (**2016**). The Oral Microbiota. **Adv Exp Med Biol** 902(45-60. doi: 10.1007/978-3-319-31248-4_4.
27. Deo PN, Deshmukh R (**2019**). Oral microbiome: Unveiling the fundamentals. **J Oral Maxillofac Pathol** 23(1): 122-128. doi: 10.4103/jomfp.JOMFP_304_18.
28. Wang M, Ahrne S, Jeppsson B, Molin G (**2005**). Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. **FEMS Microbiol Ecol** 54(2): 219-231. doi: 10.1016/j.femsec.2005.03.012.
29. Ahmed S, Macfarlane GT, Fite A, McBain AJ, Gilbert P, Macfarlane S (**2007**). Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. **Appl Environ Microbiol** 73(22): 7435-7442. doi: 10.1128/AEM.01143-07.
30. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y (**2005**). Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. **J Med Microbiol** 54(Pt 11): 1093-1101. doi: 10.1099/jmm.0.45935-0.
31. Kastl AJ, Jr., Terry NA, Wu GD, Albenberg LG (**2020**). The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. **Cell Mol Gastroenterol Hepatol** 9(1): 33-45. doi: 10.1016/j.jcmgh.2019.07.006.
32. Suhr MJ, Banjara N, Hallen-Adams HE (**2016**). Sequence-based methods for detecting and evaluating the human gut mycobiome. **Lett Appl Microbiol** 62(3): 209-215. doi: 10.1111/lam.12539.
33. Nash AK, Auchtung TA, Wong MC, Smith DP, Gesell JR, Ross MC, Stewart CJ, Metcalf GA, Muzny DM, Gibbs RA, Ajami NJ, Petrosino JF (**2017**). The gut mycobiome of the Human Microbiome Project healthy cohort. **Microbiome** 5(1): 153. doi: 10.1186/s40168-017-0373-4.
34. Belvoncikova P, Splichalova P, Videnska P, Gardlik R (**2022**). The Human Mycobiome: Colonization, Composition and the Role in Health and Disease. **J Fungi (Basel)** 8(10). doi: 10.3390/jof8101046.
35. Li Q, Wang C, Zhang Q, Tang C, Li N, Ruan B, Li J (**2012**). Use of 18S ribosomal DNA polymerase chain reaction-denaturing gradient gel electrophoresis to study composition of fungal community in 2 patients with intestinal transplants. **Hum Pathol** 43(8): 1273-1281. doi: 10.1016/j.humpath.2011.09.017.
36. Mutlu EA, Gillevet PM, Rangwala H, Sikaroodi M, Naqvi A, Engen PA, Kwasny M, Lau CK, Keshavarzian A (**2012**). Colonic microbiome is altered in alcoholism. **Am J Physiol Gastrointest Liver Physiol** 302(9): G966-978. doi: 10.1152/ajpgi.00380.2011.

37. Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L (**2008**). Comparative analysis of human gut microbiota by barcoded pyrosequencing. **PLoS One** 3(7): e2836. doi: 10.1371/journal.pone.0002836.
38. Chattopadhyay I, Dhar R, Pethusamy K, Seethy A, Srivastava T, Sah R, Sharma J, Karmakar S (**2021**). Exploring the Role of Gut Microbiome in Colon Cancer. **Appl Biochem Biotechnol** 193(6): 1780-1799. doi: 10.1007/s12010-021-03498-9.
39. Hillman ET, Lu H, Yao T, Nakatsu CH (**2017**). Microbial Ecology along the Gastrointestinal Tract. **Microbes Environ** 32(4): 300-313. doi: 10.1264/jsme2.ME17017.
40. Kelly BJ, Imai I, Bittinger K, Laughlin A, Fuchs BD, Bushman FD, Collman RG (**2016**). Composition and dynamics of the respiratory tract microbiome in intubated patients. **Microbiome** 4(7. doi: 10.1186/s40168-016-0151-8.
41. Dickson RP, Erb-Downward JR, Martinez FJ, Huffnagle GB (**2016**). The Microbiome and the Respiratory Tract. **Annu Rev Physiol** 78(481-504. doi: 10.1146/annurev-physiol-021115-105238.
42. Grice EA, Segre JA (**2011**). The skin microbiome. **Nat Rev Microbiol** 9(4): 244-253. doi: 10.1038/nrmicro2537.
43. Byrd AL, Belkaid Y, Segre JA (**2018**). The human skin microbiome. **Nat Rev Microbiol** 16(3): 143-155. doi: 10.1038/nrmicro.2017.157.
44. Grice EA, Segre JA (**2012**). The human microbiome: our second genome. **Annu Rev Genomics Hum Genet** 13(151-170. doi: 10.1146/annurev-genom-090711-163814.
45. Pohl HG, Groah SL, Perez-Losada M, Ljungberg I, Sprague BM, Chandal N, Caldovic L, Hsieh M (**2020**). The Urine Microbiome of Healthy Men and Women Differs by Urine Collection Method. **Int Neurourol J** 24(1): 41-51. doi: 10.5213/inj.1938244.122.
46. Li JKM, Chiu PKF, Ng CF (**2019**). The impact of microbiome in urological diseases: a systematic review. **Int Urol Nephrol** 51(10): 1677-1697. doi: 10.1007/s11255-019-02225-y.
47. Fouts DE, Pieper R, Szpakowski S, Pohl H, Knoblach S, Suh MJ, Huang ST, Ljungberg I, Sprague BM, Lucas SK, Torralba M, Nelson KE, Groah SL (**2012**). Integrated next-generation sequencing of 16S rDNA and metaproteomics differentiate the healthy urine microbiome from asymptomatic bacteriuria in neuropathic bladder associated with spinal cord injury. **J Transl Med** 10(174. doi: 10.1186/1479-5876-10-174.
48. Whiteside SA, Razvi H, Dave S, Reid G, Burton JP (**2015**). The microbiome of the urinary tract--a role beyond infection. **Nat Rev Urol** 12(2): 81-90. doi: 10.1038/nrurol.2014.361.
49. Drell T, Lillsaar T, Tummeleht L, Simm J, Aaspollu A, Vain E, Saarma I, Salumets A, Donders GG, Metsis M (**2013**). Characterization of the vaginal micro- and mycobiome in asymptomatic reproductive-age Estonian women. **PLoS One** 8(1): e54379. doi: 10.1371/journal.pone.0054379.
50. Fettweis JM, Serrano MG, Brooks JP, Edwards DJ, Girerd PH, Parikh HI, Huang B, Arodz TJ, Edupuganti L, Glascock AL (**2019**). The vaginal microbiome and preterm birth. **Nature medicine** 25(6): 1012-1021. doi: 10.1038/s41591-019-0450-2.
51. Rawls M, Ellis AK (**2019**). The microbiome of the nose. **Ann Allergy Asthma Immunol** 122(1): 17-24. doi: 10.1016/j.anai.2018.05.009.
52. Dimitri-Pinheiro S, Soares R, Barata P (**2020**). The Microbiome of the Nose-Friend or Foe? **Allergy Rhinol (Providence)** 11(2152656720911605. doi: 10.1177/2152656720911605.
53. Jung WH, Croll D, Cho JH, Kim YR, Lee YW (**2015**). Analysis of the nasal vestibule mycobiome in patients with allergic rhinitis. **Mycoses** 58(3): 167-172. doi: 10.1111/myc.12296.
54. Gupta A, Bhanushali S, Karyakarte R, Joshi S, Das R, Shouche Y, Sharma A (**2023**). Mycobiome profiling of nasopharyngeal region of SARS-CoV-2 infected individuals. **Microbes Infect** 25(3): 105059. doi: 10.1016/j.micinf.2022.105059.
55. Sellart-Altisent M, Torres-Rodriguez JM, Gomez de Ana S, Alvarado-Ramirez E (**2007**). Nasal fungal microbiota in allergic and healthy subjects. **Rev Iberoam Micol** 24(2): 125-130. doi: 10.1016/s1130-1406(07)70027-x.
56. Dupuy AK, David MS, Li L, Heider TN, Peterson JD, Montano EA, Dongari-Bagtzoglou A, Diaz PI, Strausbaugh LD (**2014**). Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of *Malassezia* as a prominent commensal. **PLoS One** 9(3): e90899. doi: 10.1371/journal.pone.0090899.
57. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, Gillevet PM (**2010**). Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. **PLoS Pathog** 6(1): e1000713. doi: 10.1371/journal.ppat.1000713.
58. Baraniya D, Chen T, Nahar A, Alakwaa F, Hill J, Tellez M, Ismail A, Puri S, Al-Hebshi NN (**2020**). Supragingival mycobiome and inter-kingdom interactions in dental caries. **J Oral Microbiol** 12(1): 1729305. doi: 10.1080/20002297.2020.1729305.

59. Diaz PI, Hong BY, Dupuy AK, Strausbaugh LD (**2017**). Mining the oral mycobiome: Methods, components, and meaning. **Virulence** 8(3): 313-323. doi: 10.1080/21505594.2016.1252015.
60. Beck JM, Young VB, Huffnagle GB (**2012**). The microbiome of the lung. **Transl Res** 160(4): 258-266. doi: 10.1016/j.trsl.2012.02.005.
61. Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, Young VB, Toews GB, Curtis JL, Sundaram B, Martinez FJ, Huffnagle GB (**2011**). Analysis of the lung microbiome in the "healthy" smoker and in COPD. **PLoS One** 6(2): e16384. doi: 10.1371/journal.pone.0016384.
62. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine A, Poulter L, Pachter L, Moffatt MF, Cookson WO (**2010**). Disordered microbial communities in asthmatic airways. **PLoS One** 5(1): e8578. doi: 10.1371/journal.pone.0008578.
63. Nguyen LD, Viscogliosi E, Delhaes L (**2015**). The lung mycobiome: an emerging field of the human respiratory microbiome. **Front Microbiol** 6(89. doi: 10.3389/fmicb.2015.00089.
64. Charlson ES, Diamond JM, Bittinger K, Fitzgerald AS, Yadav A, Haas AR, Bushman FD, Collman RG (**2012**). Lung-enriched organisms and aberrant bacterial and fungal respiratory microbiota after lung transplant. **Am J Respir Crit Care Med** 186(6): 536-545. doi: 10.1164/rccm.201204-0693OC.
65. Delhaes L, Monchy S, Frealle E, Hubans C, Salleron J, Leroy S, Prevotat A, Wallet F, Wallaert B, Dei-Cas E, Sime-Ngando T, Chabe M, Viscogliosi E (**2012**). The airway microbiota in cystic fibrosis: a complex fungal and bacterial community--implications for therapeutic management. **PLoS One** 7(4): e36313. doi: 10.1371/journal.pone.0036313.
66. Leong C, Schmid B, Toi MJ, Wang J, Irudayaswamy AS, Goh JPZ, Bosshard PP, Glatz M, Dawson TL, Jr. (**2019**). Geographical and Ethnic Differences Influence Culturable Commensal Yeast Diversity on Healthy Skin. **Front Microbiol** 10(1891. doi: 10.3389/fmicb.2019.01891.
67. Keum HL, Kim H, Kim HJ, Park T, Kim S, An S, Sul WJ (**2020**). Structures of the Skin Microbiome and Mycobiome Depending on Skin Sensitivity. **Microorganisms** 8(7). doi: 10.3390/microorganisms8071032.
68. Zhu T, Duan YY, Kong FQ, Galzote C, Quan ZX (**2020**). Dynamics of Skin Mycobiome in Infants. **Front Microbiol** 11(1790. doi: 10.3389/fmicb.2020.01790.
69. von Rosenvinge EC, Song Y, White JR, Maddox C, Blanchard T, Fricke WF (**2013**). Immune status, antibiotic medication and pH are associated with changes in the stomach fluid microbiota. **ISME J** 7(7): 1354-1366. doi: 10.1038/ismej.2013.33.
70. Ackerman AL, Underhill DM (**2017**). The mycobiome of the human urinary tract: potential roles for fungi in urology. **Ann Transl Med** 5(2): 31. doi: 10.21037/atm.2016.12.69.
71. Cui L, Morris A, Ghedin E (**2013**). The human mycobiome in health and disease. **Genome Med** 5(7): 63. doi: 10.1186/gm467.
72. Holland J, Young ML, Lee O, S CAC (**2003**). Vulvovaginal carriage of yeasts other than *Candida albicans*. **Sex Transm Infect** 79(3): 249-250. doi: 10.1136/sti.79.3.249.
73. Papaemmanouil V, Georgogiannis N, Plega M, Lalaki J, Lydakis D, Dimitriou M, Papadimitriou A (**2011**). Prevalence and susceptibility of *Saccharomyces cerevisiae* causing vaginitis in Greek women. **Anaerobe** 17(6): 298-299. doi: 10.1016/j.anaerobe.2011.04.008.
74. Bradford LL, Ravel J (**2017**). The vaginal mycobiome: A contemporary perspective on fungi in women's health and diseases. **Virulence** 8(3): 342-351. doi: 10.1080/21505594.2016.1237332.
75. Hallen-Adams HE, Suhr MJ (**2017**). Fungi in the healthy human gastrointestinal tract. **Virulence** 8(3): 352-358. doi: 10.1080/21505594.2016.1247140.
76. Tipton L, Ghedin E, Morris A (**2017**). The lung mycobiome in the next-generation sequencing era. **Virulence** 8(3): 334-341. doi: 10.1080/21505594.2016.1235671.
77. Jo JH, Kennedy EA, Kong HH (**2017**). Topographical and physiological differences of the skin mycobiome in health and disease. **Virulence** 8(3): 324-333. doi: 10.1080/21505594.2016.1249093.
78. Frau A, Kenny JG, Lenzi L, Campbell BJ, Ijaz UZ, Duckworth CA, Burkitt MD, Hall N, Anson J, Darby AC, Probert CSJ (**2019**). DNA extraction and amplicon production strategies deeply inf luence the outcome of gut mycobiome studies. **Sci Rep** 9(1): 9328. doi: 10.1038/s41598-019-44974-x.
79. De Filippis F, Laiola M, Blaiotta G, Ercolini D (**2017**). Different Amplicon Targets for Sequencing-Based Studies of Fungal Diversity. **Appl Environ Microbiol** 83(17). doi: 10.1128/AEM.00905-17.
80. Diaz PI, Dongari-Bagtzoglou A (**2021**). Critically Appraising the Significance of the Oral Mycobiome. **J Dent Res** 100(2): 133-140. doi: 10.1177/0022034520956975.

81. Guo R, Zheng N, Lu H, Yin H, Yao J, Chen Y (**2012**). Increased diversity of fungal flora in the vagina of patients with recurrent vaginal candidiasis and allergic rhinitis. **Microb Ecol** 64(4): 918-927. doi: 10.1007/s00248-012-0084-0.
82. Paul AA, Hoffman KL, Hagan JL, Sampath V, Petrosino JF, Pammi M (**2020**). Fungal cutaneous microbiome and host determinants in preterm and term neonates. **Pediatr Res** 88(2): 225-233. doi: 10.1038/s41390-019-0719-7.
83. Atiencia-Carrera MB, Cabezas-Mera FS, Tejera E, Machado A (**2022**). Prevalence of biofilms in *Candida* spp. bloodstream infections: A meta-analysis. **PLoS One** 17(2): e0263522. doi: 10.1371/journal.pone.0263522.
84. Cheung GYC, Bae JS, Otto M (**2021**). Pathogenicity and virulence of *Staphylococcus aureus*. **Virulence** 12(1): 547-569. doi: 10.1080/21505594.2021.1878688.
85. d'Enfert C, Kaune AK, Alaban LR, Chakraborty S, Cole N, Delavy M, Kosmala D, Marsaux B, Frois-Martins R, Morelli M, Rosati D, Valentine M, Xie Z, Emritloll Y, Warn PA, Bequet F, Bougnoux ME, Bornes S, Gresnigt MS, Hube B, Jacobsen ID, Legrand M, Leibundgut-Landmann S, Manichanh C, Munro CA, Netea MG, Queiroz K, Roget K, Thomas V, Thoral C, et al. (**2021**). The impact of the Fungus-Host-Microbiota interplay upon *Candida albicans* infections: current knowledge and new perspectives. **FEMS Microbiol Rev** 45(3). doi: 10.1093/femsre/fuaa060.
86. Vila T, Sultan AS, Montelongo-Jauregui D, Jabra-Rizk MA (**2020**). Oral Candidiasis: A Disease of Opportunity. **J Fungi (Basel)** 6(1). doi: 10.3390/jof6010015.
87. Willems HME, Ahmed SS, Liu J, Xu Z, Peters BM (**2020**). Vulvovaginal Candidiasis: A Current Understanding and Burning Questions. **J Fungi (Basel)** 6(1). doi: 10.3390/jof6010027.
88. Czechowicz P, Nowicka J, Gosciniak G (**2022**). Virulence Factors of *Candida* spp. and Host Immune Response Important in the Pathogenesis of Vulvovaginal Candidiasis. **Int J Mol Sci** 23(11). doi: 10.3390/ijms23115895.
89. Rodriguez-Cerdeira C, Martinez-Herrera E, Carnero-Gregorio M, Lopez-Barcenas A, Fabbrocini G, Fida M, El-Samahy M, Gonzalez-Cespon JL (**2020**). Pathogenesis and Clinical Relevance of *Candida* Biofilms in Vulvovaginal Candidiasis. **Front Microbiol** 11(544480. doi: 10.3389/fmicb.2020.544480.
90. Hickey DK, Patel MV, Fahey JV, Wira CR (**2011**). Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. **J Reprod Immunol** 88(2): 185-194. doi: 10.1016/j.jri.2011.01.005.
91. Ma B, Forney LJ, Ravel J (**2012**). Vaginal microbiome: rethinking health and disease. **Annu Rev Microbiol** 66(371-389. doi: 10.1146/annurev-micro-092611-150157.
92. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, De Backer E, Temmerman M, Vaneechoutte M (**2005**). Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. **BMC Microbiol** 5(61. doi: 10.1186/1471-2180-5-61.
93. Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler TL, Hall RW, Ross FJ, McCoy CO, Bumgarner R, Marrazzo JM, Fredricks DN (**2012**). Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. **PLoS One** 7(6): e37818. doi: 10.1371/journal.pone.0037818.
94. Datcu R, Gesink D, Mulvad G, Montgomery-Andersen R, Rink E, Koch A, Ahrens P, Jensen JS (**2013**). Vaginal microbiome in women from Greenland assessed by microscopy and quantitative PCR. **BMC Infect Dis** 13(480. doi: 10.1186/1471-2334-13-480.
95. Jespers V, Menten J, Smet H, Poradosu S, Abdellati S, Verhelst R, Hardy L, Buve A, Crucitti T (**2012**). Quantification of bacterial species of the vaginal microbiome in different groups of women, using nucleic acid amplification tests. **BMC Microbiol** 12(83. doi: 10.1186/1471-2180-12-83.
96. Nunn KL, Forney LJ (**2016**). Unraveling the Dynamics of the Human Vaginal Microbiome. **Yale J Biol Med** 89(3): 331-337. doi: PMID: 27698617.
97. Lamont RF, Sobel JD, Akins RA, Hassan SS, Chaiworapongsa T, Kusanovic JP, Romero R (**2011**). The vaginal microbiome: new information about genital tract flora using molecular based techniques. **BJOG** 118(5): 533-549. doi: 10.1111/j.1471-0528.2010.02840.x.
98. Lebeer S, Ahannach S, Wittouck S, Gehrmann T, Eilers T, Oerlemans E, Condori S, Dillen J, Spacova I, Vander Donck L (**2022**). Citizen-science map of the vaginal microbiome. doi: 10.21203/rs.3.rs-1350465/v1.

99. Hyman RW, Fukushima M, Diamond L, Kumm J, Giudice LC, Davis RW (**2005**). Microbes on the human vaginal epithelium. **Proc Natl Acad Sci U S A** 102(22): 7952-7957. doi: 10.1073/pnas.0503236102.
100. Fiers WD, Gao IH, Iliev ID (**2019**). Gut mycobiota under scrutiny: fungal symbionts or environmental transients? **Curr Opin Microbiol** 50(79-86. doi: 10.1016/j.mib.2019.09.010.
101. Tortelli BA, Lewis WG, Allsworth JE, Member-Meneh N, Foster LR, Reno HE, Peipert JF, Fay JC, Lewis AL (**2020**). Associations between the vaginal microbiome and *Candida* colonization in women of reproductive age. **Am J Obstet Gynecol** 222(5): 471 e471-471 e479. doi: 10.1016/j.ajog.2019.10.008.
102. Sobel JD, Chaim W (**1996**). Vaginal microbiology of women with acute recurrent vulvovaginal candidiasis. **J Clin Microbiol** 34(10): 2497-2499. doi: 10.1128/jcm.34.10.2497-2499.1996.
103. Kalia N, Singh J, Kaur M (**2020**). Microbiota in vaginal health and pathogenesis of recurrent vulvovaginal infections: a critical review. **Ann Clin Microbiol Antimicrob** 19(1): 5. doi: 10.1186/s12941-020-0347-4.
104. Miles MR, Olsen L, Rogers A (**1977**). Recurrent vaginal candidiasis. Importance of an intestinal reservoir. **JAMA** 238(17): 1836-1837. doi: 10.1001/jama.238.17.1836.
105. Amabebe E, Anumba DOC (**2020**). Female Gut and Genital Tract Microbiota-Induced Crosstalk and Differential Effects of Short-Chain Fatty Acids on Immune Sequelae. **Front Immunol** 11(2184. doi: 10.3389/fimmu.2020.02184.
106. Song SD, Acharya KD, Zhu JE, Deveney CM, Walther-Antonio MRS, Tetel MJ, Chia N (**2020**). Daily Vaginal Microbiota Fluctuations Associated with Natural Hormonal Cycle, Contraceptives, Diet, and Exercise. **mSphere** 5(4). doi: 10.1128/mSphere.00593-20.
107. Lin YP, Chen WC, Cheng CM, Shen CJ (**2021**). Vaginal pH Value for Clinical Diagnosis and Treatment of Common Vaginitis. **Diagnostics (Basel)** 11(11). doi: 10.3390/diagnostics11111996.
108. Tandon D, Patil Sr A, Goriwale M (**2022**). Effect of various hormonal and non-hormonal contraceptive methods on the vaginal milieu-A narrative review. **medRxiv**: 2022.2005. 2017.22275192. doi: 10.1101/2022.05.17.22275192.
109. Linhares IM, Summers PR, Larsen B, Giraldo PC, Witkin SS (**2011**). Contemporary perspectives on vaginal pH and lactobacilli. **Am J Obstet Gynecol** 204(2): 120 e121-125. doi: 10.1016/j.ajog.2010.07.010.
110. Zhou X, Bent SJ, Schneider MG, Davis CC, Islam MR, Forney LJ (**2004**). Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. **Microbiology (Reading)** 150(Pt 8): 2565-2573. doi: 10.1099/mic.0.26905-0.
111. Newton ER, Piper JM, Shain RN, Perdue ST, Peairs W (**2001**). Predictors of the vaginal microflora. **Am J Obstet Gynecol** 184(5): 845-853; discussion 853-845. doi: 10.1067/mob.2001.113848.
112. Priestley CJ, Jones BM, Dhar J, Goodwin L (**1997**). What is normal vaginal flora? **Genitourin Med** 73(1): 23-28. doi: 10.1136/sti.73.1.23.
113. Farage M, Maibach H (**2006**). Lifetime changes in the vulva and vagina. **Arch Gynecol Obstet** 273(4): 195-202. doi: 10.1007/s00404-005-0079-x.
114. Hammerschlag MR, Alpert S, Rosner I, Thurston P, Semine D, McComb D, McCormack WM (**1978**). Microbiology of the vagina in children: normal and potentially pathogenic organisms. **Pediatrics** 62(1): 57-62. doi: PMID: 98750.
115. Hammerschlag MR, Alpert S, Onderdonk AB, Thurston P, Drude E, McCormack WM, Bartlett JG (**1978**). Anaerobic microflora of the vagina in children. **Am J Obstet Gynecol** 131(8): 853-856. doi: 10.1016/s0002-9378(16)33130-1.
116. Jaquiery A, Stylianopoulos A, Hogg G, Grover S (**1999**). Vulvovaginitis: clinical features, aetiology, and microbiology of the genital tract. **Archives of disease in childhood** 81(1): 64-67. doi: 10.1136/adc.81.1.64.
117. Farage MA, Miller KW, Sobel JD (**2010**). Dynamics of the vaginal ecosystem—hormonal influences. **Infectious Diseases: Research and Treatment** 3(IDRT. S3903. doi: 10.4137/idrt.s3903.
118. Shafer MA, Sweet RL, Ohm-Smith MJ, Shalwitz J, Beck A, Schachter J (**1985**). Microbiology of the lower genital tract in postmenarchal adolescent girls: differences by sexual activity, contraception, and presence of nonspecific vaginitis. **J Pediatr** 107(6): 974-981. doi: 10.1016/s0022-3476(85)80208-0.
119. Galhardo C, Soares Jr J, Simões R, Haidar M, Rodrigues de Lima G, Baracat E (**2006**). Estrogen effects on the vaginal pH, flora and cytology in late postmenopause after a long period without hormone therapy. **Clinical and experimental obstetrics & gynecology** 33(2): 85-89. doi: PMID: 16903243.

120. Gupta S, Kumar N, Singhal N, Kaur R, Manektala U (**2006**). Vaginal microflora in postmenopausal women on hormone replacement therapy. **Indian journal of pathology & microbiology** 49(3): 457-461. doi: PMID: 17001923.
121. Cauci S, Driussi S, De Santo D, Penacchioni P, Iannicelli T, Lanzafame P, De Seta F, Quadrifoglio F, de Aloysio D, Guaschino S (**2002**). Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. **J Clin Microbiol** 40(6): 2147-2152. doi: 10.1128/JCM.40.6.2147-2152.2002.
122. Hillier SL, Lau RJ (**1997**). Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. **Clin Infect Dis** 25 Suppl 2(S123-126. doi: 10.1086/516221.
123. Crucitti T, Hardy L, van de Wijgert J, Agaba S, Buyze J, Kestelyn E, Delvaux T, Mwambarangwe L, De Baetselier I, Jespers V, Ring Plus study g (**2018**). Contraceptive rings promote vaginal lactobacilli in a high bacterial vaginosis prevalence population: A randomised, open-label longitudinal study in Rwandan women. **PLoS One** 13(7): e0201003. doi: 10.1371/journal.pone.0201003.
124. van de Wijgert JH, Verwijs MC, Turner AN, Morrison CS (**2013**). Hormonal contraception decreases bacterial vaginosis but oral contraception may increase candidiasis: implications for HIV transmission. **AIDS** 27(13): 2141-2153. doi: 10.1097/QAD.0b013e32836290b6.
125. Hyman RW, Herndon CN, Jiang H, Palm C, Fukushima M, Bernstein D, Vo KC, Zelenko Z, Davis RW, Giudice LC (**2012**). The dynamics of the vaginal microbiome during infertility therapy with *in vitro* fertilization-embryo transfer. **J Assist Reprod Genet** 29(2): 105-115. doi: 10.1007/s10815-011-9694-6.
126. Jakobsson T, Forsum U (**2008**). Changes in the predominant human *Lactobacillus* flora during *in vitro* fertilisation. **Ann Clin Microbiol Antimicrob** 7(14. doi: 10.1186/1476-0711-7-14.
127. Chaban B, Links MG, Jayaprakash TP, Wagner EC, Bourque DK, Lohn Z, Albert AY, van Schalkwyk J, Reid G, Hemmingsen SM, Hill JE, Money DM (**2014**). Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. **Microbiome** 2(23. doi: 10.1186/2049-2618-2-23.
128. Amabebe E, Anumba DO (**2018**). The vaginal microenvironment: the physiologic role of lactobacilli. **Frontiers in medicine** 5(181. doi: 10.3389/fmed.2018.00181.
129. Juliana NCA, Suiters MJM, Al-Nasiry S, Morre SA, Peters RPH, Ambrosino E (**2020**). The Association Between Vaginal Microbiota Dysbiosis, Bacterial Vaginosis, and Aerobic Vaginitis, and Adverse Pregnancy Outcomes of Women Living in Sub-Saharan Africa: A Systematic Review. **Front Public Health** 8(567885. doi: 10.3389/fpubh.2020.567885.
130. Ceccarani C, Foschi C, Parolin C, D'Antuono A, Gaspari V, Consolandi C, Laghi L, Camboni T, Vitali B, Severgnini M, Marangoni A (**2019**). Diversity of vaginal microbiome and metabolome during genital infections. **Sci Rep** 9(1): 14095. doi: 10.1038/s41598-019-50410-x.
131. Hummelen R, Fernandes AD, Macklaim JM, Dickson RJ, Changalucha J, Gloor GB, Reid G (**2010**). Deep sequencing of the vaginal microbiota of women with HIV. **PLoS One** 5(8): e12078. doi: 10.1371/journal.pone.0012078.
132. Mastromarino P, Macchia S, Meggiorini L, Trinchieri V, Mosca L, Perluigi M, Midulla C (**2009**). Effectiveness of *Lactobacillus*-containing vaginal tablets in the treatment of symptomatic bacterial vaginosis. **Clin Microbiol Infect** 15(1): 67-74. doi: 10.1111/j.1469-0691.2008.02112.x.
133. Tamrakar R, Yamada T, Furuta I, Cho K, Morikawa M, Yamada H, Sakuragi N, Minakami H (**2007**). Association between Lactobacillus species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. **BMC Infect Dis** 7(128. doi: 10.1186/1471-2334-7-128.
134. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S (**2015**). *Lactobacillus* species as biomarkers and agents that can promote various aspects of vaginal health. **Front Physiol** 6(81. doi: 10.3389/fphys.2015.00081.
135. Parolin C, Foschi C, Laghi L, Zhu C, Banzola N, Gaspari V, D'Antuono A, Giordani B, Severgnini M, Consolandi C, Salvo M, Cevenini R, Vitali B, Marangoni A (**2018**). Insights Into Vaginal Bacterial Communities and Metabolic Profiles of *Chlamydia trachomatis* Infection: Positioning Between Eubiosis and Dysbiosis. **Front Microbiol** 9(600. doi: 10.3389/fmicb.2018.00600.
136. Liu MB, Xu SR, He Y, Deng GH, Sheng HF, Huang XM, Ouyang CY, Zhou HW (**2013**). Diverse vaginal microbiomes in reproductive-age women with vulvovaginal candidiasis. **PLoS One** 8(11): e79812. doi: 10.1371/journal.pone.0079812.

137. Zhou X, Westman R, Hickey R, Hansmann MA, Kennedy C, Osborn TW, Forney LJ (**2009**). Vaginal microbiota of women with frequent vulvovaginal candidiasis. **Infect Immun** 77(9): 4130-4135. doi: 10.1128/IAI.00436-09.
138. Osset J, Garcia E, Bartolome R, Andreu A (**2001**). Role of *Lactobacillus* as protector against vaginal candidiasis. **Medicina clinica** 117(8): 285-288. doi: 10.1016/s0025-7753(01)72089-1
139. Jian S, SU M-j (**2015**). Features of vaginal bacteria community in women with recurrent vulvovaginal candidiasis. **Journal of Reproduction and Contraception** 26(4): 229-238. doi: 10.7669/j.issn.1001-7844.2015.04.0229.
140. McKloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, Thomas R, Richardson R, Gerasimidis K, Nile CJ, Williams C, Ramage G (**2021**). Recurrent Vulvovaginal Candidiasis: a Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*. **mSystems** 6(4): e0062221. doi: 10.1128/mSystems.00622-21.
141. Bloom SM, Mafunda NA, Woolston BM, Hayward MR, Frempong JF, Abai AB, Xu J, Mitchell AJ, Westergaard X, Hussain FA, Xulu N, Dong M, Dong KL, Gumbi T, Ceasar FX, Rice JK, Choksi N, Ismail N, Ndung'u T, Ghebremichael MS, Relman DA, Balskus EP, Mitchell CM, Kwon DS (**2022**). Cysteine dependence of *Lactobacillus iners* is a potential therapeutic target for vaginal microbiota modulation. **Nat Microbiol** 7(3): 434-450. doi: 10.1038/s41564-022-01070-7.
142. Hollister EB, Riehle K, Luna RA, Weidler EM, Rubio-Gonzales M, Mistretta TA, Raza S, Doddapaneni HV, Metcalf GA, Muzny DM, Gibbs RA, Petrosino JF, Shulman RJ, Versalovic J (**2015**). Structure and function of the healthy pre-adolescent pediatric gut microbiome. **Microbiome** 3(36. doi: 10.1186/s40168-015-0101-x.
143. McBurney MI, Davis C, Fraser CM, Schneeman BO, Huttenhower C, Verbeke K, Walter J, Latulippe ME (**2019**). Establishing What Constitutes a Healthy Human Gut Microbiome: State of the Science, Regulatory Considerations, and Future Directions. **J Nutr** 149(11): 1882-1895. doi: 10.1093/jn/nxz154.
144. Lloyd-Price J, Mahurkar A, Rahnavard G, Crabtree J, Orvis J, Hall AB, Brady A, Creasy HH, McCracken C, Giglio MG, McDonald D, Franzosa EA, Knight R, White O, Huttenhower C (**2017**). Strains, functions and dynamics in the expanded Human Microbiome Project. **Nature** 550(7674): 61-66. doi: 10.1038/nature23889.
145. Falony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, Kurilshikov A, Bonder MJ, Valles-Colomer M, Vandeputte D, Tito RY, Chaffron S, Rymenans L, Verspecht C, De Sutter L, Lima-Mendez G, D'Hoe K, Jonckheere K, Homola D, Garcia R, Tigchelaar EF, Eeckhaudt L, Fu J, Henckaerts L, Zhernakova A, Wijmenga C, Raes J (**2016**). Population-level analysis of gut microbiome variation. **Science** 352(6285): 560-564. doi: 10.1126/science.aad3503.
146. Human Microbiome Project C (**2012**). Structure, function and diversity of the healthy human microbiome. **Nature** 486(7402): 207-214. doi: 10.1038/nature11234.
147. Sundin OH, Mendoza-Ladd A, Zeng M, Diaz-Arévalo D, Morales E, Fagan BM, Ordoñez J, Velez P, Antony N, McCallum RW (**2017**). The human jejunum has an endogenous microbiota that differs from those in the oral cavity and colon. **BMC microbiology** 17(1): 1-17. doi: 10.1186/s12866-017-1059-6.
148. Vasapolli R, Schutte K, Schulz C, Vital M, Schomburg D, Pieper DH, Vilchez-Vargas R, Malfertheiner P (**2019**). Analysis of Transcriptionally Active Bacteria Throughout the Gastrointestinal Tract of Healthy Individuals. **Gastroenterology** 157(4): 1081-1092 e1083. doi: 10.1053/j.gastro.2019.05.068.
149. Derrien M, van Hylckama Vlieg JE (**2015**). Fate, activity, and impact of ingested bacteria within the human gut microbiota. **Trends Microbiol** 23(6): 354-366. doi: 10.1016/j.tim.2015.03.002.
150. Reuter G (**2001**). The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. **Current issues in intestinal microbiology** 2(2): 43-53. doi: PMID: 11721280.
151. Rossi M, Martinez-Martinez D, Amaretti A, Ulrici A, Raimondi S, Moya A (**2016**). Mining metagenomic whole genome sequences revealed subdominant but constant *Lactobacillus* population in the human gut microbiota. **Environ Microbiol Rep** 8(3): 399-406. doi: 10.1111/1758-2229.12405.
152. Dal Bello F, Walter J, Hammes WP, Hertel C (**2003**). Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition. **Microb Ecol** 45(4): 455-463. doi: 10.1007/s00248-003-2001-z.
153. Ahrne S, Lonnermark E, Wold AE, Aberg N, Hesselmar B, Saalman R, Strannegard IL, Molin G, Adlerberth I (**2005**). Lactobacilli in the intestinal microbiota of Swedish infants. **Microbes Infect** 7(11-12): 1256-1262. doi: 10.1016/j.micinf.2005.04.011.

154. Lonnermark E, Nowrouzinan F, Adlerberth I, Ahrne S, Wold A, Friman V (**2012**). Oral and faecal lactobacilli and their expression of mannose-specific adhesins in individuals with and without IgA deficiency. **Int J Med Microbiol** 302(1): 53-60. doi: 10.1016/j.ijmm.2011.08.004.
155. Ozturk M, Meterelliyoz M (**2015**). Practical identification of human originated *Lactobacillus* species by amplified ribosomal DNA restriction analysis (ARDRA) for probiotic use. **Mol Biol Rep** 42(8): 1323-1332. doi: 10.1007/s11033-015-3877-7.
156. Delgado S, Suárez A, Mayo B (**2007**). Dominant cultivable *Lactobacillus* species from the feces of healthy adults in northern Spain. **International Microbiology** 10(2): 141. doi: PMID: 17661293.
157. Badet C, Thebaud NB (**2008**). Ecology of lactobacilli in the oral cavity: a review of literature. **Open Microbiol J** 2(38-48. doi: 10.2174/1874285800802010038.
158. Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, Lewis JD, Bushman FD (**2013**). Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. **PLoS One** 8(6): e66019. doi: 10.1371/journal.pone.0066019.
159. Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, Mujagic Z, Vila AV, Falony G, Vieira-Silva S, Wang J, Imhann F, Brandsma E, Jankipersadsing SA, Joossens M, Cenit MC, Deelen P, Swertz MA, LifeLines cohort s, Weersma RK, Feskens EJ, Netea MG, Gevers D, Jonkers D, Franke L, Aulchenko YS, Huttenhower C, Raes J, Hofker MH, Xavier RJ, et al. (**2016**). Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. **Science** 352(6285): 565-569. doi: 10.1126/science.aad3369.
160. Backhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, Li Y, Xia Y, Xie H, Zhong H, Khan MT, Zhang J, Li J, Xiao L, Al-Aama J, Zhang D, Lee YS, Kotowska D, Colding C, Tremaroli V, Yin Y, Bergman S, Xu X, Madsen L, Kristiansen K, Dahlgren J, Wang J (**2015**). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. **Cell Host Microbe** 17(5): 690-703. doi: 10.1016/j.chom.2015.04.004.
161. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, Ley RE (**2014**). Human genetics shape the gut microbiome. **Cell** 159(4): 789-799. doi: 10.1016/j.cell.2014.09.053.
162. Wang S, Charbonnier LM, Noval Rivas M, Georgiev P, Li N, Gerber G, Bry L, Chatila TA (**2015**). MyD88 Adaptor-Dependent Microbial Sensing by Regulatory T Cells Promotes Mucosal Tolerance and Enforces Commensalism. **Immunity** 43(2): 289-303. doi: 10.1016/j.immuni.2015.06.014.
163. Maurice CF, Haiser HJ, Turnbaugh PJ (**2013**). Xenobiotics shape the physiology and gene expression of the active human gut microbiome. **Cell** 152(1-2): 39-50. doi: 10.1016/j.cell.2012.10.052.
164. Hsiao A, Ahmed AM, Subramanian S, Griffin NW, Drewry LL, Petri WA, Jr., Haque R, Ahmed T, Gordon JI (**2014**). Members of the human gut microbiota involved in recovery from *Vibrio cholerae* infection. **Nature** 515(7527): 423-426. doi: 10.1038/nature13738.
165. Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, Abramson L, Katz MN, Korem T, Zmora N, Kuperman Y, Biton I, Gilad S, Harmelin A, Shapiro H, Halpern Z, Segal E, Elinav E (**2014**). Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. **Cell** 159(3): 514-529. doi: 10.1016/j.cell.2014.09.048.
166. Fujimura KE, Demoor T, Rauch M, Faruqi AA, Jang S, Johnson CC, Boushey HA, Zoratti E, Ownby D, Lukacs NW, Lynch SV (**2014**). House dust exposure mediates gut microbiome *Lactobacillus* enrichment and airway immune defense against allergens and virus infection. **Proc Natl Acad Sci U S A** 111(2): 805-810. doi: 10.1073/pnas.1310750111.
167. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI (**2012**). Human gut microbiome viewed across age and geography. **Nature** 486(7402): 222-227. doi: 10.1038/nature11053.
168. Schanche M, Avershina E, Dotterud C, Oien T, Storro O, Johnsen R, Rudi K (**2015**). High-Resolution Analyses of Overlap in the Microbiota Between Mothers and Their Children. **Curr Microbiol** 71(2): 283-290. doi: 10.1007/s00284-015-0843-5.
169. Thompson SC, Greville HS, Param R (**2008**). Beyond policy and planning to practice: getting sexual health on the agenda in Aboriginal communities in Western Australia. **Aust New Zealand Health Policy** 5(3. doi: 10.1186/1743-8462-5-3.
170. Ravcheev DA, Godzik A, Osterman AL, Rodionov DA (**2013**). Polysaccharides utilization in human gut bacterium *Bacteroides thetaiotaomicron*: comparative genomics reconstruction of metabolic and regulatory networks. **BMC Genomics** 14(873. doi: 10.1186/1471-2164-14-873.

171. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE (**2011**). Succession of microbial consortia in the developing infant gut microbiome. **Proc Natl Acad Sci U S A** 108 Suppl 1(Suppl 1): 4578-4585. doi: 10.1073/pnas.1000081107.
172. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA, Stobberingh EE (**2006**). Factors influencing the composition of the intestinal microbiota in early infancy. **Pediatrics** 118(2): 511-521. doi: 10.1542/peds.2005-2824.
173. Mangin I, Suau A, Gotteland M, Brunser O, Pochart P (**2010**). Amoxicillin treatment modifies the composition of *Bifidobacterium* species in infant intestinal microbiota. **Anaerobe** 16(4): 433-438. doi: 10.1016/j.anaerobe.2010.06.005.
174. Nermes M, Endo A, Aarnio J, Salminen S, Isolauri E (**2015**). Furry pets modulate gut microbiota composition in infants at risk for allergic disease. **J Allergy Clin Immunol** 136(6): 1688-1690 e1681. doi: 10.1016/j.jaci.2015.07.029.
175. Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, Aguilera M, Khanna S, Gil A, Edwards CA, Dore J, Other Members of the IT (**2010**). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. **J Pediatr Gastroenterol Nutr** 51(1): 77-84. doi: 10.1097/MPG.0b013e3181d1b11e.
176. Cheng J, Ringel-Kulka T, Heikamp-de Jong I, Ringel Y, Carroll I, de Vos WM, Salojarvi J, Satokari R (**2016**). Discordant temporal development of bacterial phyla and the emergence of core in the fecal microbiota of young children. **ISME J** 10(4): 1002-1014. doi: 10.1038/ismej.2015.177.
177. Saulnier DM, Riehle K, Mistretta TA, Diaz MA, Mandal D, Raza S, Weidler EM, Qin X, Coarfa C, Milosavljevic A, Petrosino JF, Highlander S, Gibbs R, Lynch SV, Shulman RJ, Versalovic J (**2011**). Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. **Gastroenterology** 141(5): 1782-1791. doi: 10.1053/j.gastro.2011.06.072.
178. Shin JH, Park YH, Sim M, Kim SA, Joung H, Shin DM (**2019**). Serum level of sex steroid hormone is associated with diversity and profiles of human gut microbiome. **Res Microbiol** 170(4-5): 192-201. doi: 10.1016/j.resmic.2019.03.003.
179. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD (**2011**). Linking long-term dietary patterns with gut microbial enterotypes. **Science** 334(6052): 105-108. doi: 10.1126/science.1208344.
180. Zhu A, Sunagawa S, Mende DR, Bork P (**2015**). Inter-individual differences in the gene content of human gut bacterial species. **Genome Biol** 16(1): 82. doi: 10.1186/s13059-015-0646-9.
181. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Dore J, Corthier G, Furet JP (**2009**). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. **BMC Microbiol** 9(123. doi: 10.1186/1471-2180-9-123.
182. van Tongeren SP, Slaets JP, Harmsen HJ, Welling GW (**2005**). Fecal microbiota composition and frailty. **Appl Environ Microbiol** 71(10): 6438-6442. doi: 10.1128/AEM.71.10.6438-6442.2005.
183. Rampelli S, Schnorr SL, Consolandi C, Turroni S, Severgnini M, Peano C, Brigidi P, Crittenden AN, Henry AG, Candela M (**2015**). Metagenome Sequencing of the Hadza Hunter-Gatherer Gut Microbiota. **Curr Biol** 25(13): 1682-1693. doi: 10.1016/j.cub.2015.04.055.
184. Nakayama J, Watanabe K, Jiang J, Matsuda K, Chao SH, Haryono P, La-Ongkham O, Sarwoko MA, Sujaya IN, Zhao L, Chen KT, Chen YP, Chiu HH, Hidaka T, Huang NX, Kiyohara C, Kurakawa T, Sakamoto N, Sonomoto K, Tashiro K, Tsuji H, Chen MJ, Leelavatcharamas V, Liao CC, Nitisinprasert S, Rahayu ES, Ren FZ, Tsai YC, Lee YK (**2015**). Diversity in gut bacterial community of school-age children in Asia. **Sci Rep** 5(8397. doi: 10.1038/srep08397.
185. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW (**2012**). Gut microbiota composition correlates with diet and health in the elderly. **Nature** 488(7410): 178-184. doi: 10.1038/nature11319.
186. Lax S, Smith DP, Hampton-Marcell J, Owens SM, Handley KM, Scott NM, Gibbons SM, Larsen P, Shogan BD, Weiss S, Metcalf JL, Ursell LK, Vazquez-Baeza Y, Van Treuren W, Hasan NA, Gibson MK, Colwell R, Dantas G, Knight R, Gilbert JA (**2014**). Longitudinal analysis of microbial interaction between humans and the indoor environment. **Science** 345(6200): 1048-1052. doi: 10.1126/science.1254529.

187. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von Bergen M, McCoy KD, Macpherson AJ, Danska JS (**2013**). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. **Science** 339(6123): 1084-1088. doi: 10.1126/science.1233521.
188. Kuo SM (**2013**). The interplay between fiber and the intestinal microbiome in the inflammatory response. **Adv Nutr** 4(1): 16-28. doi: 10.3945/an.112.003046.
189. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P (**2010**). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. **Proc Natl Acad Sci U S A** 107(33): 14691-14696. doi: 10.1073/pnas.1005963107.
190. Beaugerie L, Petit JC (**2004**). Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. **Best Pract Res Clin Gastroenterol** 18(2): 337-352. doi: 10.1016/j.bpg.2003.10.002.
191. Dethlefsen L, Huse S, Sogin ML, Relman DA (**2008**). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. **PLoS Biol** 6(11): e280. doi: 10.1371/journal.pbio.0060280.
192. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, van Sinderen D, O'Connor M, Harnedy N, O'Connor K, Henry C, O'Mahony D, Fitzgerald AP, Shanahan F, Twomey C, Hill C, Ross RP, O'Toole PW (**2011**). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. **Proc Natl Acad Sci U S A** 108 Suppl 1(Suppl 1): 4586-4591. doi: 10.1073/pnas.1000097107.
193. Schmidt TSB, Raes J, Bork P (**2018**). The Human Gut Microbiome: From Association to Modulation. **Cell** 172(6): 1198-1215. doi: 10.1016/j.cell.2018.02.044.
194. Schirmer M, Franzosa EA, Lloyd-Price J, McIver LJ, Schwager R, Poon TW, Ananthakrishnan AN, Andrews E, Barron G, Lake K, Prasad M, Sauk J, Stevens B, Wilson RG, Braun J, Denson LA, Kugathasan S, McGovern DPB, Vlamakis H, Xavier RJ, Huttenhower C (**2018**). Dynamics of metatranscription in the inflammatory bowel disease gut microbiome. **Nat Microbiol** 3(3): 337-346. doi: 10.1038/s41564-017-0089-z.
195. Akobeng AK, Singh P, Kumar M, Al Khodor S (**2020**). Role of the gut microbiota in the pathogenesis of coeliac disease and potential therapeutic implications. **Eur J Nutr** 59(8): 3369-3390. doi: 10.1007/s00394-020-02324-y.
196. Caminero A, Herran AR, Nistal E, Perez-Andres J, Vaquero L, Vivas S, Ruiz de Morales JM, Albillos SM, Casqueiro J (**2014**). Diversity of the cultivable human gut microbiome involved in gluten metabolism: isolation of microorganisms with potential interest for coeliac disease. **FEMS Microbiol Ecol** 88(2): 309-319. doi: 10.1111/1574-6941.12295.
197. Zhao W, Ho HE, Bunyavanich S (**2019**). The gut microbiome in food allergy. **Ann Allergy Asthma Immunol** 122(3): 276-282. doi: 10.1016/j.anai.2018.12.012.
198. Yoshida N, Yamashita T, Hirata KI (**2018**). Gut Microbiome and Cardiovascular Diseases. **Diseases** 6(3). doi: 10.3390/diseases6030056.
199. Tseng CH, Wu CY (**2019**). The gut microbiome in obesity. **J Formos Med Assoc** 118 Suppl 1(S3-S9. doi: 10.1016/j.jfma.2018.07.009.
200. Amrane S, Hocquart M, Afouda P, Kuete E, Pham TP, Dione N, Ngom, II, Valles C, Bachar D, Raoult D, Lagier JC (**2019**). Metagenomic and culturomic analysis of gut microbiota dysbiosis during *Clostridium difficile* infection. **Sci Rep** 9(1): 12807. doi: 10.1038/s41598-019-49189-8.
201. Wang D, Li Y, Zhong H, Ding Q, Lin Y, Tang S, Zong Y, Wang Q, Zhang X, Yang H, Wang R, Liu X (**2019**). Alterations in the human gut microbiome associated with *Helicobacter pylori* infection. **FEBS Open Bio** 9(9): 1552-1560. doi: 10.1002/2211-5463.12694.
202. Inoue T, Nakayama J, Moriya K, Kawaratani H, Momoda R, Ito K, Iio E, Nojiri S, Fujiwara K, Yoneda M, Yoshiji H, Tanaka Y (**2018**). Gut Dysbiosis Associated With Hepatitis C Virus Infection. **Clin Infect Dis** 67(6): 869-877. doi: 10.1093/cid/ciy205.
203. Venzon M, Bernard-Raichon L, Klein J, Axelrad JE, Zhang C, Hussey GA, Sullivan AP, Casanovas-Massana A, Noval MG, Valero-Jimenez AM, Gago J, Putzel G, Pironti A, Wilder E, Yale IRT, Thorpe LE, Littman DR, Dittmann M, Stapleford KA, Shopsin B, Torres VJ, Ko AI, Iwasaki A, Cadwell K, Schluter J (**2022**). Gut microbiome dysbiosis during COVID-19 is associated with increased risk for bacteremia and microbial translocation. **bioRxiv**. doi: 10.1101/2021.07.15.452246.
204. Sokol H, Jegou S, McQuitty C, Straub M, Leducq V, Landman C, Kirchgesner J, Le Gall G, Bourrier A, Nion-Larmurier I, Cosnes J, Seksik P, Richard ML, Beaugerie L (**2018**). Specificities

of the intestinal microbiota in patients with inflammatory bowel disease and *Clostridium difficile* infection. **Gut Microbes** 9(1): 55-60. doi: 10.1080/19490976.2017.1361092.
205. Debast SB, Bauer MP, Kuijper EJ, European Society of Clinical M, Infectious D (**2014**). European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection. **Clin Microbiol Infect** 20 Suppl 2(1-26. doi: 10.1111/1469-0691.12418.
206. Gallardo P, Izquierdo M, Vidal RM, Chamorro-Veloso N, Rossello-Mora R, O'Ryan M, Farfan MJ (**2017**). Distinctive Gut Microbiota Is Associated with Diarrheagenic *Escherichia coli* Infections in Chilean Children. **Front Cell Infect Microbiol** 7(424. doi: 10.3389/fcimb.2017.00424.
207. Sghir A, Gramet G, Suau A, Rochet V, Pochart P, Dore J (**2000**). Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. **Appl Environ Microbiol** 66(5): 2263-2266. doi: 10.1128/AEM.66.5.2263-2266.2000.
208. Nistal E, Caminero A, Herran AR, Perez-Andres J, Vivas S, Ruiz de Morales JM, Saenz de Miera LE, Casqueiro J (**2016**). Study of duodenal bacterial communities by 16S rRNA gene analysis in adults with active celiac disease vs non-celiac disease controls. **J Appl Microbiol** 120(6): 1691-1700. doi: 10.1111/jam.13111.
209. Liu HN, Wu H, Chen YZ, Chen YJ, Shen XZ, Liu TT (**2017**). Altered molecular signature of intestinal microbiota in irritable bowel syndrome patients compared with healthy controls: A systematic review and meta-analysis. **Dig Liver Dis** 49(4): 331-337. doi: 10.1016/j.dld.2017.01.142.
210. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, Luckey DH, Marietta EV, Jeraldo PR, Chen X, Weinshenker BG, Rodriguez M, Kantarci OH, Nelson H, Murray JA, Mangalam AK (**2016**). Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. **Sci Rep** 6(28484. doi: 10.1038/srep28484.
211. Borges-Canha M, Portela-Cidade JP, Dinis-Ribeiro M, Leite-Moreira AF, Pimentel-Nunes P (**2015**). Role of colonic microbiota in colorectal carcinogenesis: a systematic review. **Revista Española de Enfermedades Digestivas** 107(11): 659-671. doi: 10.17235/reed.2015.3830/2015.
212. Lewis JD, Chen EZ, Baldassano RN, Otley AR, Griffiths AM, Lee D, Bittinger K, Bailey A, Friedman ES, Hoffmann C, Albenberg L, Sinha R, Compher C, Gilroy E, Nessel L, Grant A, Chehoud C, Li H, Wu GD, Bushman FD (**2015**). Inflammation, Antibiotics, and Diet as Environmental Stressors of the Gut Microbiome in Pediatric Crohn's Disease. **Cell Host Microbe** 18(4): 489-500. doi: 10.1016/j.chom.2015.09.008.
213. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, Wu X, Li J, Tang L, Li Y (**2015**). The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. **Nature medicine** 21(8): 895-905. doi: 10.1038/nm.3914.
214. Hieken TJ, Chen J, Hoskin TL, Walther-Antonio M, Johnson S, Ramaker S, Xiao J, Radisky DC, Knutson KL, Kalari KR (**2016**). The microbiome of aseptically collected human breast tissue in benign and malignant disease. **Scientific reports** 6(1): 30751. doi: 10.1038/srep30751.
215. Guerrero-Preston R, Godoy-Vitorino F, Jedlicka A, Rodríguez-Hilario A, González H, Bondy J, Lawson F, Folawiyo O, Michailidi C, Dziedzic A (**2016**). 16S rRNA amplicon sequencing identifies microbiota associated with oral cancer, human papilloma virus infection and surgical treatment. **Oncotarget** 7(32): 51320. doi: 10.18632/oncotarget.9710.
216. Lauer E, Kandler O (**1980**). *Lactobacillus gasseri* sp. nov., a new species of the subgenus *Thermobacterium*. **Zentralblatt für Bakteriologie: I Abt Originale C: Allgemeine, angewandte und ökologische Mikrobiologie** 1(1): 75-78. doi: 10.1016/S0172-5564(80)80019-4.
217. Ito Y, Kawai Y, Arakawa K, Honme Y, Sasaki T, Saito T (**2009**). Conjugative plasmid from *Lactobacillus gasseri* LA39 that carries genes for production of and immunity to the circular bacteriocin gassericin A. **Appl Environ Microbiol** 75(19): 6340-6351. doi: 10.1128/AEM.00195-09.
218. Scillato M, Spitale A, Mongelli G, Privitera GF, Mangano K, Cianci A, Stefani S, Santagati M (**2021**). Antimicrobial properties of *Lactobacillus* cell-free supernatants against multidrug-resistant urogenital pathogens. **Microbiologyopen** 10(2): e1173. doi: 10.1002/mbo3.1173.
219. Garcia-Gutierrez E, O'Connor PM, Colquhoun IJ, Vior NM, Rodriguez JM, Mayer MJ, Cotter PD, Narbad A (**2020**). Production of multiple bacteriocins, including the novel bacteriocin gassericin M, by *Lactobacillus gasseri* LM19, a strain isolated from human milk. **Appl Microbiol Biotechnol** 104(9): 3869-3884. doi: 10.1007/s00253-020-10493-3.
220. Strus M, Brzychczy-Wloch M, Gosiewski T, Kochan P, Heczko PB (**2006**). The *in vitro* effect of hydrogen peroxide on vaginal microbial communities. **FEMS Immunol Med Microbiol** 48(1): 56-63. doi: 10.1111/j.1574-695X.2006.00120.x.

221. Morais IMC, Cordeiro AL, Teixeira GS, Domingues VS, Nardi RMD, Monteiro AS, Alves RJ, Siqueira EP, Santos VL (**2017**). Biological and physicochemical properties of biosurfactants produced by *Lactobacillus jensenii* P(6A) and *Lactobacillus gasseri* P(65). **Microb Cell Fact** 16(1): 155. doi: 10.1186/s12934-017-0769-7.
222. Azcarate-Peril MA, Altermann E, Goh YJ, Tallon R, Sanozky-Dawes RB, Pfeiler EA, O'Flaherty S, Buck BL, Dobson A, Duong T, Miller MJ, Barrangou R, Klaenhammer TR (**2008**). Analysis of the genome sequence of *Lactobacillus gasseri* ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism. **Appl Environ Microbiol** 74(15): 4610-4625. doi: 10.1128/AEM.00054-08.
223. Sungur T, Aslim B, Karaaslan C, Aktas B (**2017**). Impact of Exopolysaccharides (EPSs) of *Lactobacillus gasseri* strains isolated from human vagina on cervical tumor cells (HeLa). **Anaerobe** 47(137-144. doi: 10.1016/j.anaerobe.2017.05.013.
224. Kandler O, Stetter K-O, Köhl R (**1980**). *Lactobacillus reuteri* sp. nov., a new species of heterofermentative lactobacilli. **Zentralblatt Für Bakteriologie: I Abt Originale C: Allgemeine, Angewandte Und Ökologische Mikrobiologie** 1(3): 264-269. doi: 10.1016/S0172-5564(80)80007-8.
225. Riad AM, Widyarman AS (**2021**). The Effect of Parabiotic Reuterin on the Expression of Genes Involved in *Candida albicans* Biofilm Formation: An *Ex vivo* Study. **Journal of Dentistry Indonesia** 28(3): 163-170. doi: 10.14693/jdi.v28i3.1276.
226. Widyarman AS, Maukar TV, Tjandrawinata R, Theodora CF (**2021**). Antibiofilm Activity of Parabiotic Reuterin on Acrylic Resin Plates. **2021 IEEE International Conference on Health, Instrumentation & Measurement, and Natural Sciences (InHeNce)**: 1-6. doi: 10.1109/InHeNce52833.2021.9537250.
227. Ganzle MG, Holtzel A, Walter J, Jung G, Hammes WP (**2000**). Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. **Appl Environ Microbiol** 66(10): 4325-4333. doi: 10.1128/AEM.66.10.4325-4333.2000.
228. Ganzle MG, Vogel RF (**2003**). Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. **Int J Food Microbiol** 80(1): 31-45. doi: 10.1016/s0168-1605(02)00146-0.
229. Kabuki T, Saito T, Kawai Y, Uemura J, Itoh T (**1997**). Production, purification and characterization of reutericin 6, a bacteriocin with lytic activity produced by *Lactobacillus reuteri* LA6. **Int J Food Microbiol** 34(2): 145-156. doi: 10.1016/s0168-1605(96)01180-4.
230. Kawai Y, Ishii Y, Uemura K, Kitazawa H, Saito T, Itoh T (**2001**). *Lactobacillus reuteri* LA6 and *Lactobacillus gasseri* LA39 isolated from faeces of the same human infant produce identical cyclic bacteriocin. **Food microbiology** 18(4): 407-415. doi: 10.1006/fmic.2001.0412.
231. Sun MC, Hu ZY, Li DD, Chen YX, Xi JH, Zhao CH (**2022**). Application of the Reuterin System as Food Preservative or Health-Promoting Agent: A Critical Review. **Foods** 11(24). doi: 10.3390/foods11244000.
232. Morita H, Toh H, Fukuda S, Horikawa H, Oshima K, Suzuki T, Murakami M, Hisamatsu S, Kato Y, Takizawa T, Fukuoka H, Yoshimura T, Itoh K, O'Sullivan DJ, McKay LL, Ohno H, Kikuchi J, Masaoka T, Hattori M (**2008**). Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. **DNA Res** 15(3): 151-161. doi: 10.1093/dnares/dsn009.
233. Ciandrini E, Campana R, Casettari L, Perinelli DR, Fagioli L, Manti A, Palmieri GF, Papa S, Baffone W (**2016**). Characterization of biosurfactants produced by *Lactobacillus* spp. and their activity against oral streptococci biofilm. **Appl Microbiol Biotechnol** 100(15): 6767-6777. doi: 10.1007/s00253-016-7531-7.
234. Salehi R, Savabi O, Kazemi M, Kamali S, Salehi AR, Eslami G, Tahmourespour A (**2014**). Effects of *Lactobacillus reuteri*-derived biosurfactant on the gene expression profile of essential adhesion genes (gtfB, gtfC and ftf) of *Streptococcus mutans*. **Adv Biomed Res** 3(169. doi: 10.4103/2277-9175.139134.
235. Kang MS, Oh JS, Lee HC, Lim HS, Lee SW, Yang KH, Choi NK, Kim SM (**2011**). Inhibitory effect of *Lactobacillus reuteri* on periodontopathic and cariogenic bacteria. **J Microbiol** 49(2): 193-199. doi: 10.1007/s12275-011-0252-9.
236. Jorgensen MR, Kragelund C, Jensen PO, Keller MK, Twetman S (**2017**). Probiotic *Lactobacillus reuteri* has antifungal effects on oral *Candida* species *in vitro*. **J Oral Microbiol** 9(1): 1274582. doi: 10.1080/20002297.2016.1274582.
237. Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K (**2004**). Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. **Appl Environ Microbiol** 70(2): 1176-1181. doi: 10.1128/AEM.70.2.1176-1181.2004.

238. Hemarajata P, Gao C, Pflughoeft KJ, Thomas CM, Saulnier DM, Spinler JK, Versalovic J (**2013**). *Lactobacillus reuteri*-specific immunoregulatory gene rsiR modulates histamine production and immunomodulation by *Lactobacillus reuteri*. **J Bacteriol** 195(24): 5567-5576. doi: 10.1128/JB.00261-13.
239. Greifova G, Majekova H, Greif G, Body P, Greifova M, Dubnickova M (**2017**). Analysis of antimicrobial and immunomodulatory substances produced by heterofermentative *Lactobacillus reuteri*. **Folia Microbiol (Praha)** 62(6): 515-524. doi: 10.1007/s12223-017-0524-9.
240. MacKenzie DA, Jeffers F, Parker ML, Vibert-Vallet A, Bongaerts RJ, Roos S, Walter J, Juge N (**2010**). Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of *Lactobacillus reuteri*. **Microbiology (Reading)** 156(Pt 11): 3368-3378. doi: 10.1099/mic.0.043265-0.
241. Gunning AP, Kavanaugh D, Thursby E, Etzold S, MacKenzie DA, Juge N (**2016**). Use of Atomic Force Microscopy to Study the Multi-Modular Interaction of Bacterial Adhesins to Mucins. **Int J Mol Sci** 17(11). doi: 10.3390/ijms17111854.
242. Deo D, Davray D, Kulkarni R (**2019**). A Diverse Repertoire of Exopolysaccharide Biosynthesis Gene Clusters in *Lactobacillus* Revealed by Comparative Analysis in 106 Sequenced Genomes. **Microorganisms** 7(10). doi: 10.3390/microorganisms7100444.
243. Suokko A, Poutanen M, Savijoki K, Kalkkinen N, Varmanen P (**2008**). ClpL is essential for induction of thermotolerance and is potentially part of the HrcA regulon in *Lactobacillus gasseri*. **Proteomics** 8(5): 1029-1041. doi: 10.1002/pmic.200700925.
244. Wall T, Bath K, Britton RA, Jonsson H, Versalovic J, Roos S (**2007**). The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. **Appl Environ Microbiol** 73(12): 3924-3935. doi: 10.1128/AEM.01502-06.
245. Pfeiler EA, Klaenhammer TR (**2009**). Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. **Appl Environ Microbiol** 75(18): 6013-6016. doi: 10.1128/AEM.00495-09.
246. Whitehead K, Versalovic J, Roos S, Britton RA (**2008**). Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. **Appl Environ Microbiol** 74(6): 1812-1819. doi: 10.1128/AEM.02259-07.
247. Giri S, Kindo AJ (**2012**). A review of *Candida* species causing blood stream infection. **Indian J Med Microbiol** 30(3): 270-278. doi: 10.4103/0255-0857.99484.
248. Suhr MJ, Hallen-Adams HE (**2015**). The human gut mycobiome: pitfalls and potentials--a mycologist's perspective. **Mycologia** 107(6): 1057-1073. doi: 10.3852/15-147.
249. Barousse MM, Van Der Pol BJ, Fortenberry D, Orr D, Fidel PL, Jr. (**2004**). Vaginal yeast colonisation, prevalence of vaginitis, and associated local immunity in adolescents. **Sex Transm Infect** 80(1): 48-53. doi: 10.1136/sti.2002.003855.
250. Sobel JD (**2007**). Vulvovaginal candidosis. **Lancet** 369(9577): 1961-1971. doi: 10.1016/S0140-6736(07)60917-9.
251. Corsello S, Spinillo A, Osnengo G, Penna C, Guaschino S, Beltrame A, Blasi N, Festa A (**2003**). An epidemiological survey of vulvovaginal candidiasis in Italy. **Eur J Obstet Gynecol Reprod Biol** 110(1): 66-72. doi: 10.1016/s0301-2115(03)00096-4.
252. Das I, Nightingale P, Patel M, Jumaa P (**2011**). Epidemiology, clinical characteristics, and outcome of candidemia: experience in a tertiary referral center in the UK. **Int J Infect Dis** 15(11): e759-763. doi: 10.1016/j.ijid.2011.06.006.
253. Almirante B, Rodriguez D, Park BJ, Cuenca-Estrella M, Planes AM, Almela M, Mensa J, Sanchez F, Ayats J, Gimenez M, Saballs P, Fridkin SK, Morgan J, Rodriguez-Tudela JL, Warnock DW, Pahissa A, Barcelona Candidemia Project Study G (**2005**). Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: results from population-based surveillance, barcelona, Spain, from 2002 to 2003. **J Clin Microbiol** 43(4): 1829-1835. doi: 10.1128/JCM.43.4.1829-1835.2005.
254. Zhang W, Song X, Wu H, Zheng R (**2019**). Epidemiology, risk factors and outcomes of *Candida albicans* vs. non-albicans candidaemia in adult patients in Northeast China. **Epidemiol Infect** 147(e277. doi: 10.1017/S0950268819001638.
255. Boyd Tressler A, Markwei M, Fortin C, Yao M, Procop GW, Soper DE, Goje O (**2021**). Risks for Recurrent Vulvovaginal Candidiasis Caused by Non-*Albicans* Candida Versus *Candida Albicans*. **J Womens Health (Larchmt)** 30(11): 1588-1596. doi: 10.1089/jwh.2020.8811.
256. Goncalves B, Ferreira C, Alves CT, Henriques M, Azeredo J, Silva S (**2016**). Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. **Crit Rev Microbiol** 42(6): 905-927. doi: 10.3109/1040841X.2015.1091805.

257. Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN (**2019**). Twenty Years of the SENTRY Antifungal Surveillance Program: Results for *Candida* Species From 1997-2016. **Open Forum Infect Dis** 6(Suppl 1): S79-S94. doi: 10.1093/ofid/ofy358.
258. Gangneux JP, Cornet M, Bailly S, Fradin C, Feger C, Timsit JF, Leroy O, Sendid B, Bougnoux ME (**2018**). Clinical Impact of Antifungal Susceptibility, Biofilm Formation and Mannoside Expression of *Candida* Yeasts on the Outcome of Invasive Candidiasis in ICU: An Ancillary Study on the Prospective AmarCAND2 Cohort. **Front Microbiol** 9(2907. doi: 10.3389/fmicb.2018.02907.
259. Salazar SB, Simoes RS, Pedro NA, Pinheiro MJ, Carvalho M, Mira NP (**2020**). An Overview on Conventional and Non-Conventional Therapeutic Approaches for the Treatment of Candidiasis and Underlying Resistance Mechanisms in Clinical Strains. **J Fungi (Basel)** 6(1). doi: 10.3390/jof6010023.
260. Raad I, Hanna H, Boktour M, Girgawy E, Danawi H, Mardani M, Kontoyiannis D, Darouiche R, Hachem R, Bodey GP (**2004**). Management of central venous catheters in patients with cancer and candidemia. **Clin Infect Dis** 38(8): 1119-1127. doi: 10.1086/382874.
261. Ghrenassia E, Mokart D, Mayaux J, Demoule A, Rezine I, Kerhuel L, Calvet L, De Jong A, Azoulay E, Darmon M (**2019**). Candidemia in critically ill immunocompromised patients: report of a retrospective multicenter cohort study. **Ann Intensive Care** 9(1): 62. doi: 10.1186/s13613-019-0539-2.
262. Sozio E, Pieralli F, Azzini AM, Tintori G, Demma F, Furneri G, Sbrana F, Bertolino G, Fortunato S, Meini S, Bragantini D, Morettini A, Nozzoli C, Menichetti F, Concia E, Tascini C, Group GFCS (**2018**). A prediction rule for early recognition of patients with candidemia in Internal Medicine: results from an Italian, multicentric, case-control study. **Infection** 46(5): 625-633. doi: 10.1007/s15010-018-1162-0.
263. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB (**2004**). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. **Clin Infect Dis** 39(3): 309-317. doi: 10.1086/421946.
264. Aljeboori Z, Gorelik A, Jenkins E, McFarlane T, Darvall J (**2018**). Risk factors for candidaemia and their cumulative effect over time in a cohort of critically ill, non-neutropenic patients. **Critical Care and Resuscitation** 20(4): 313-319. doi: PMID: 30482139.
265. Mazi PB, Olsen MA, Stwalley D, Rauseo AM, Ayres C, Powderly WG, Spec A (**2022**). Attributable Mortality of *Candida* Bloodstream Infections in the Modern Era: A Propensity Score Analysis. **Clin Infect Dis** 75(6): 1031-1036. doi: 10.1093/cid/ciac004.
266. Lee RA, Zurko JC, Camins BC, Griffin RL, Rodriguez JM, McCarty TP, Magadia J, Pappas PG (**2019**). Impact of Infectious Disease Consultation on Clinical Management and Mortality in Patients With Candidemia. **Clin Infect Dis** 68(9): 1585-1587. doi: 10.1093/cid/ciy849.
267. Lausch KR, Sogaard M, Rosenvinge FS, Johansen HK, Boysen T, Roder B, Mortensen KL, Nielsen L, Lemming L, Olesen B, Leitz C, Kristensen L, Dzajic E, Ostergaard L, Schonheyder HC, Arendrup MC (**2018**). High incidence of candidaemia in a nationwide cohort: Underlying diseases, risk factors and mortality. **Int J Infect Dis** 76(58-63. doi: 10.1016/j.ijid.2018.08.010.
268. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ (**2018**). Invasive candidiasis. **Nat Rev Dis Primers** 4(18026. doi: 10.1038/nrdp.2018.26.
269. Tortorano AM, Prigitano A, Morroni G, Brescini L, Barchiesi F (**2021**). Candidemia: Evolution of Drug Resistance and Novel Therapeutic Approaches. **Infect Drug Resist** 14(5543-5553. doi: 10.2147/IDR.S274872.
270. Naglik JR, Richardson JP, Moyes DL (**2014**). *Candida albicans* pathogenicity and epithelial immunity. **PLoS Pathog** 10(8): e1004257. doi: 10.1371/journal.ppat.1004257.
271. Wachtler B, Wilson D, Hube B (**2011**). *Candida albicans* adhesion to and invasion and damage of vaginal epithelial cells: stage-specific inhibition by clotrimazole and bifonazole. **Antimicrob Agents Chemother** 55(9): 4436-4439. doi: 10.1128/AAC.00144-11.
272. Klempp-Selb B, Rimek D, Kappe R (**2000**). Karyotyping of *Candida albicans* and *Candida glabrata* from patients with *Candida* sepsis. **Mycoses** 43(5): 159-163. doi: 10.1046/j.1439-0507.2000.00555.x.
273. Spence D (**2010**). Candidiasis (vulvovaginal). **BMJ clinical evidence** 2010(doi: PMID: 21718579.
274. Achkar JM, Fries BC (**2010**). *Candida* infections of the genitourinary tract. **Clin Microbiol Rev** 23(2): 253-273. doi: 10.1128/CMR.00076-09.
275. Pappas PG, Kauffman CA, Andes D, Benjamin DK, Jr., Calandra TF, Edwards JE, Jr., Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD, Infectious Diseases Society of A (**2009**). Clinical practice guidelines for the management of

candidiasis: 2009 update by the Infectious Diseases Society of America. **Clin Infect Dis** 48(5): 503-535. doi: 10.1086/596757.
276. Sobel JD, Faro S, Force RW, Foxman B, Ledger WJ, Nyirjesy PR, Reed BD, Summers PR (**1998**). Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. **Am J Obstet Gynecol** 178(2): 203-211. doi: 10.1016/s0002-9378(98)80001-x.
277. Anderson MR, Klink K, Cohrssen A (**2004**). Evaluation of vaginal complaints. **JAMA** 291(11): 1368-1379. doi: 10.1001/jama.291.11.1368.
278. Mitchell H (**2004**). Vaginal discharge--causes, diagnosis, and treatment. **BMJ** 328(7451): 1306-1308. doi: 10.1136/bmj.328.7451.1306.
279. Sobel JD (**1997**). Vaginitis. **N Engl J Med** 337(26): 1896-1903. doi: 10.1056/NEJM199712253372607.
280. Liu H (**2002**). Co-regulation of pathogenesis with dimorphism and phenotypic switching in *Candida albicans*, a commensal and a pathogen. **Int J Med Microbiol** 292(5-6): 299-311. doi: 10.1078/1438-4221-00215.
281. Peters BM, Yano J, Noverr MC, Fidel PL, Jr. (**2014**). *Candida* vaginitis: when opportunism knocks, the host responds. **PLoS Pathog** 10(4): e1003965. doi: 10.1371/journal.ppat.1003965.
282. Patel DA, Gillespie B, Sobel JD, Leaman D, Nyirjesy P, Weitz MV, Foxman B (**2004**). Risk factors for recurrent vulvovaginal candidiasis in women receiving maintenance antifungal therapy: results of a prospective cohort study. **Am J Obstet Gynecol** 190(3): 644-653. doi: 10.1016/j.ajog.2003.11.027.
283. Seider K, Heyken A, Luttich A, Miramon P, Hube B (**2010**). Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape. **Curr Opin Microbiol** 13(4): 392-400. doi: 10.1016/j.mib.2010.05.001.
284. Gilbert AS, Wheeler RT, May RC (**2014**). Fungal Pathogens: Survival and Replication within Macrophages. **Cold Spring Harb Perspect Med** 5(7): a019661. doi: 10.1101/cshperspect.a019661.
285. Fesel PH, Zuccaro A (**2016**). beta-glucan: Crucial component of the fungal cell wall and elusive MAMP in plants. **Fungal Genet Biol** 90(53-60. doi: 10.1016/j.fgb.2015.12.004.
286. Klis FM, Boorsma A, De Groot PW (**2006**). Cell wall construction in *Saccharomyces cerevisiae*. **Yeast** 23(3): 185-202. doi: 10.1002/yea.1349.
287. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S (**2003**). Dectin-1 mediates the biological effects of beta-glucans. **J Exp Med** 197(9): 1119-1124. doi: 10.1084/jem.20021890.
288. Wheeler RT, Fink GR (**2006**). A drug-sensitive genetic network masks fungi from the immune system. **PLoS Pathog** 2(4): e35. doi: 10.1371/journal.ppat.0020035.
289. Ballou ER, Avelar GM, Childers DS, Mackie J, Bain JM, Wagener J, Kastora SL, Panea MD, Hardison SE, Walker LA, Erwig LP, Munro CA, Gow NA, Brown GD, MacCallum DM, Brown AJ (**2016**). Lactate signalling regulates fungal beta-glucan masking and immune evasion. **Nat Microbiol** 2(16238. doi: 10.1038/nmicrobiol.2016.238.
290. Kaur R, Ma B, Cormack BP (**2007**). A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. **Proc Natl Acad Sci U S A** 104(18): 7628-7633. doi: 10.1073/pnas.0611195104.
291. West L, Lowman DW, Mora-Montes HM, Grubb S, Murdoch C, Thornhill MH, Gow NA, Williams D, Haynes K (**2013**). Differential virulence of *Candida glabrata* glycosylation mutants. **J Biol Chem** 288(30): 22006-22018. doi: 10.1074/jbc.M113.478743.
292. Seider K, Gerwien F, Kasper L, Allert S, Brunke S, Jablonowski N, Schwarzmuller T, Barz D, Rupp S, Kuchler K, Hube B (**2014**). Immune evasion, stress resistance, and efficient nutrient acquisition are crucial for intracellular survival of *Candida glabrata* within macrophages. **Eukaryot Cell** 13(1): 170-183. doi: 10.1128/EC.00262-13.
293. Seider K, Brunke S, Schild L, Jablonowski N, Wilson D, Majer O, Barz D, Haas A, Kuchler K, Schaller M, Hube B (**2011**). The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. **J Immunol** 187(6): 3072-3086. doi: 10.4049/jimmunol.1003730.
294. Kasper L, Seider K, Gerwien F, Allert S, Brunke S, Schwarzmuller T, Ames L, Zubiria-Barrera C, Mansour MK, Becken U, Barz D, Vyas JM, Reiling N, Haas A, Haynes K, Kuchler K, Hube B (**2014**). Identification of *Candida glabrata* genes involved in pH modulation and modification of the phagosomal environment in macrophages. **PLoS One** 9(5): e96015. doi: 10.1371/journal.pone.0096015.
295. Bain JM, Louw J, Lewis LE, Okai B, Walls CA, Ballou ER, Walker LA, Reid D, Munro CA, Brown AJ, Brown GD, Gow NA, Erwig LP (**2014**). *Candida albicans* hypha formation and mannan

masking of beta-glucan inhibit macrophage phagosome maturation. **mBio** 5(6): e01874. doi: 10.1128/mBio.01874-14.
296. Bain J, Gow NA, Erwig LP (**2015**). Novel insights into host-fungal pathogen interactions derived from live-cell imaging. **Semin Immunopathol** 37(2): 131-139. doi: 10.1007/s00281-014-0463-3.
297. Segal BH, Grimm MJ, Khan AN, Han W, Blackwell TS (**2012**). Regulation of innate immunity by NADPH oxidase. **Free Radic Biol Med** 53(1): 72-80. doi: 10.1016/j.freeradbiomed.2012.04.022.
298. Wellington M, Dolan K, Krysan DJ (**2009**). Live *Candida albicans* suppresses production of reactive oxygen species in phagocytes. **Infect Immun** 77(1): 405-413. doi: 10.1128/IAI.00860-08.
299. Roetzer A, Klopf E, Gratz N, Marcet-Houben M, Hiller E, Rupp S, Gabaldon T, Kovarik P, Schuller C (**2011**). Regulation of *Candida glabrata* oxidative stress resistance is adapted to host environment. **FEBS Lett** 585(2): 319-327. doi: 10.1016/j.febslet.2010.12.006.
300. Roetzer A, Gratz N, Kovarik P, Schuller C (**2010**). Autophagy supports *Candida glabrata* survival during phagocytosis. **Cell Microbiol** 12(2): 199-216. doi: 10.1111/j.1462-5822.2009.01391.x.
301. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J (**2012**). *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. **FEMS Microbiol Rev** 36(2): 288-305. doi: 10.1111/j.1574-6976.2011.00278.x.
302. Balakrishnan SN, Yamang H, Lorenz MC, Chew SY, Than LTL (**2022**). Role of Vaginal Mucosa, Host Immunity and Microbiota in Vulvovaginal Candidiasis. **Pathogens** 11(6). doi: 10.3390/pathogens11060618.
303. Demirezen S, Dirlik O, Beksac M (**2005**). The association of *Candida* infection with intrauterine contraceptive device. **Cent Eur J Public Health** 13(1): 32-34. doi: PMID: 15859178.
304. Verstrepen KJ, Klis FM (**2006**). Flocculation, adhesion and biofilm formation in yeasts. **Mol Microbiol** 60(1): 5-15. doi: 10.1111/j.1365-2958.2006.05072.x.
305. Chaffin WL (**2008**). *Candida albicans* cell wall proteins. **Microbiol Mol Biol Rev** 72(3): 495-544. doi: 10.1128/MMBR.00032-07.
306. Hoyer LL, Green CB, Oh SH, Zhao X (**2008**). Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family--a sticky pursuit. **Med Mycol** 46(1): 1-15. doi: 10.1080/13693780701435317.
307. Cormack BP, Ghori N, Falkow S (**1999**). An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. **Science** 285(5427): 578-582. doi: 10.1126/science.285.5427.578.
308. Alves CT, Wei XQ, Silva S, Azeredo J, Henriques M, Williams DW (**2014**). *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. **J Infect** 69(4): 396-407. doi: 10.1016/j.jinf.2014.06.002.
309. de Souza CM, Dos Santos MM, Furlaneto-Maia L, Furlaneto MC (**2023**). Adhesion and biofilm formation by the opportunistic pathogen *Candida tropicalis*: what do we know? **Can J Microbiol**. doi: 10.1139/cjm-2022-0195.
310. Hoyer LL, Fundyga R, Hecht JE, Kapteyn JC, Klis FM, Arnold J (**2001**). Characterization of agglutinin-like sequence genes from non-albicans *Candida* and phylogenetic analysis of the ALS family. **Genetics** 157(4): 1555-1567. doi: 10.1093/genetics/157.4.1555.
311. Bertini A, Zoppo M, Lombardi L, Rizzato C, De Carolis E, Vella A, Torelli R, Sanguinetti M, Tavanti A (**2016**). Targeted gene disruption in *Candida parapsilosis* demonstrates a role for CPAR2_404800 in adhesion to a biotic surface and in a murine model of ascending urinary tract infection. **Virulence** 7(2): 85-97. doi: 10.1080/21505594.2015.1112491.
312. Jayatilake JA, Samaranayake YH, Cheung LK, Samaranayake LP (**2006**). Quantitative evaluation of tissue invasion by wild type, hyphal and SAP mutants of *Candida albicans*, and non-albicans *Candida* species in reconstituted human oral epithelium. **J Oral Pathol Med** 35(8): 484-491. doi: 10.1111/j.1600-0714.2006.00435.x.
313. Hostetter MK (**1994**). Adhesins and ligands involved in the interaction of *Candida* spp. with epithelial and endothelial surfaces. **Clin Microbiol Rev** 7(1): 29-42. doi: 10.1128/CMR.7.1.29.
314. Hube B, Sanglard D, Odds FC, Hess D, Monod M, Schafer W, Brown AJ, Gow NA (**1997**). Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. **Infect Immun** 65(9): 3529-3538. doi: 10.1128/iai.65.9.3529-3538.1997.
315. Taylor BN, Staib P, Binder A, Biesemeier A, Sehnal M, Rollinghoff M, Morschhauser J, Schroppel K (**2005**). Profile of *Candida albicans*-secreted aspartic proteinase elicited during vaginal infection. **Infect Immun** 73(3): 1828-1835. doi: 10.1128/IAI.73.3.1828-1835.2005.

316. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL (**2005**). *Candida* biofilms: an update. **Eukaryot Cell** 4(4): 633-638. doi: 10.1128/EC.4.4.633-638.2005.
317. Sandai D, Tabana YM, Ouweini AE, Ayodeji IO (**2016**). Resistance of *Candida albicans* Biofilms to Drugs and the Host Immune System. **Jundishapur J Microbiol** 9(11): e37385. doi: 10.5812/jjm.37385.
318. Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J (**2009**). Our current understanding of fungal biofilms. **Crit Rev Microbiol** 35(4): 340-355. doi: 10.3109/10408410903241436.
319. Alim D, Sircaik S, Panwar SL (**2018**). The Significance of Lipids to Biofilm Formation in *Candida albicans*: An Emerging Perspective. **J Fungi (Basel)** 4(4). doi: 10.3390/jof4040140.
320. Silva S, Henriques M, Martins A, Oliveira R, Williams D, Azeredo J (**2009**). Biofilms of non-*Candida* albicans *Candida* species: quantification, structure and matrix composition. **Med Mycol** 47(7): 681-689. doi: 10.3109/13693780802549594.
321. Jain N, Kohli R, Cook E, Gialanella P, Chang T, Fries BC (**2007**). Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. **Appl Environ Microbiol** 73(6): 1697-1703. doi: 10.1128/AEM.02439-06.
322. Harriott MM, Lilly EA, Rodriguez TE, Fidel PL, Noverr MC (**2010**). *Candida albicans* forms biofilms on the vaginal mucosa. **Microbiology (Reading)** 156(Pt 12): 3635-3644. doi: 10.1099/mic.0.039354-0.
323. Wu X, Zhang S, Li H, Shen L, Dong C, Sun Y, Chen H, Xu B, Zhuang W, Deighton M, Qu Y (**2020**). Biofilm Formation of *Candida albicans* Facilitates Fungal Infiltration and Persister Cell Formation in Vaginal Candidiasis. **Front Microbiol** 11(1117. doi: 10.3389/fmicb.2020.01117.
324. Chassot F, Negri MF, Svidzinski AE, Donatti L, Peralta RM, Svidzinski TI, Consolaro ME (**2008**). Can intrauterine contraceptive devices be a *Candida albicans* reservoir? **Contraception** 77(5): 355-359. doi: 10.1016/j.contraception.2008.01.007.
325. Schaller M, Borelli C, Korting HC, Hube B (**2005**). Hydrolytic enzymes as virulence factors of *Candida albicans*. **Mycoses** 48(6): 365-377. doi: 10.1111/j.1439-0507.2005.01165.x.
326. Monika S, Malgorzata B, Zbigniew O (**2017**). Contribution of Aspartic Proteases in *Candida* Virulence. Protease Inhibitors against *Candida* Infections. **Curr Protein Pept Sci** 18(10): 1050-1062. doi: 10.2174/1389203717666160809155749.
327. Odds FC (**2008**). Secreted proteinases and *Candida albicans* virulence. **Microbiology (Reading)** 154(Pt 11): 3245-3246. doi: 10.1099/mic.0.2008/023671-0.
328. Merkerova M, Dostal J, Hradilek M, Pichova I, Hruskova-Heidingsfeldova O (**2006**). Cloning and characterization of Sapp2p, the second aspartic proteinase isoenzyme from *Candida parapsilosis*. **FEMS Yeast Res** 6(7): 1018-1026. doi: 10.1111/j.1567-1364.2006.00142.x.
329. Zaugg C, Borg-Von Zepelin M, Reichard U, Sanglard D, Monod M (**2001**). Secreted aspartic proteinase family of *Candida tropicalis*. **Infect Immun** 69(1): 405-412. doi: 10.1128/IAI.69.1.405-412.2001.
330. Hassan Y, Chew SY, Than LTL (**2021**). *Candida glabrata*: Pathogenicity and Resistance Mechanisms for Adaptation and Survival. **J Fungi (Basel)** 7(8). doi: 10.3390/jof7080667.
331. Chakrabarti A, Nayak N, Talwar P (**1991**). *In vitro* proteinase production by *Candida* species. **Mycopathologia** 114(3): 163-168. doi: 10.1007/BF00437209.
332. Koelsch G, Tang J, Loy JA, Monod M, Jackson K, Foundling SI, Lin X (**2000**). Enzymic characteristics of secreted aspartic proteases of *Candida albicans*. **Biochim Biophys Acta** 1480(1-2): 117-131. doi: 10.1016/s0167-4838(00)00068-6.
333. O'Hanlon DE, Moench TR, Cone RA (**2013**). Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. **PLoS One** 8(11): e80074. doi: 10.1371/journal.pone.0080074.
334. Ozcan SK, Budak F, Yucesoy G, Susever S, Willke A (**2006**). Prevalence, susceptibility profile and proteinase production of yeasts causing vulvovaginitis in Turkish women. **APMIS** 114(2): 139-145. doi: 10.1111/j.1600-0463.2006.apm_360.x.
335. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J (**2011**). Adherence and biofilm formation of non-*Candida albicans Candida* species. **Trends Microbiol** 19(5): 241-247. doi: 10.1016/j.tim.2011.02.003.
336. Ghannoum MA (**2000**). Potential role of phospholipases in virulence and fungal pathogenesis. **Clin Microbiol Rev** 13(1): 122-143, table of contents. doi: 10.1128/CMR.13.1.122.
337. Mohandas V, Ballal M (**2011**). Distribution of *Candida* species in different clinical samples and their virulence: biofilm formation, proteinase and phospholipase production: a study on hospitalized patients in southern India. **J Glob Infect Dis** 3(1): 4-8. doi: 10.4103/0974-777X.77288.

338. Samaranayake YH, Dassanayake RS, Cheung BP, Jayatilake JA, Yeung KW, Yau JY, Samaranayake LP (**2006**). Differential phospholipase gene expression by *Candida albicans* in artificial media and cultured human oral epithelium. **APMIS** 114(12): 857-866. doi: 10.1111/j.1600-0463.2006.apm_479.x.
339. Naglik JR, Rodgers CA, Shirlaw PJ, Dobbie JL, Fernandes-Naglik LL, Greenspan D, Agabian N, Challacombe SJ (**2003**). Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. **J Infect Dis** 188(3): 469-479. doi: 10.1086/376536.
340. Allen D, Wilson D, Drew R, Perfect J (**2015**). Azole antifungals: 35 years of invasive fungal infection management. **Expert Rev Anti Infect Ther** 13(6): 787-798. doi: 10.1586/14787210.2015.1032939.
341. Hoekstra WJ, Garvey EP, Moore WR, Rafferty SW, Yates CM, Schotzinger RJ (**2014**). Design and optimization of highly-selective fungal CYP51 inhibitors. **Bioorg Med Chem Lett** 24(15): 3455-3458. doi: 10.1016/j.bmcl.2014.05.068.
342. Odds FC, Brown AJ, Gow NA (**2003**). Antifungal agents: mechanisms of action. **Trends Microbiol** 11(6): 272-279. doi: 10.1016/s0966-842x(03)00117-3.
343. Ghannoum MA, Rice LB (**1999**). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. **Clin Microbiol Rev** 12(4): 501-517. doi: 10.1128/CMR.12.4.501.
344. Patil S, Rao RS, Majumdar B, Anil S (**2015**). Clinical Appearance of Oral *Candida* Infection and Therapeutic Strategies. **Front Microbiol** 6(1391. doi: 10.3389/fmicb.2015.01391.
345. Mayers DL, Sobel JD, Ouellette M, Kaye KS, Marchaim D (**2017**). Antimicrobial Drug Resistance: Clinical and Epidemiological Aspects, Volume 2. Springer.
346. Andes D (**2003**). *In vivo* pharmacodynamics of antifungal drugs in treatment of candidiasis. **Antimicrob Agents Chemother** 47(4): 1179-1186. doi: 10.1128/AAC.47.4.1179-1186.2003.
347. Lemke A, Kiderlen AF, Kayser O (**2005**). Amphotericin B. **Appl Microbiol Biotechnol** 68(2): 151-162. doi: 10.1007/s00253-005-1955-9.
348. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD (**2016**). Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. **Clin Infect Dis** 62(4): e1-50. doi: 10.1093/cid/civ933.
349. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC (**2012**). Hidden killers: human fungal infections. **Sci Transl Med** 4(165): 165rv113. doi: 10.1126/scitranslmed.3004404.
350. Sofjan AK, Mitchell A, Shah DN, Nguyen T, Sim M, Trojcak A, Beyda ND, Garey KW (**2018**). Rezafungin (CD101), a next-generation echinocandin: A systematic literature review and assessment of possible place in therapy. **J Glob Antimicrob Resist** 14(58-64. doi: 10.1016/j.jgar.2018.02.013.
351. Pfaller MA, Messer SA, Rhomberg PR, Castanheira M (**2017**). Activity of a Long-Acting Echinocandin (CD101) and Seven Comparator Antifungal Agents Tested against a Global Collection of Contemporary Invasive Fungal Isolates in the SENTRY 2014 Antifungal Surveillance Program. **Antimicrob Agents Chemother** 61(3). doi: 10.1128/AAC.02045-16.
352. Sandison T, Ong V, Lee J, Thye D (**2017**). Safety and Pharmacokinetics of CD101 IV, a Novel Echinocandin, in Healthy Adults. **Antimicrob Agents Chemother** 61(2). doi: 10.1128/AAC.01627-16.
353. Vermes A, Guchelaar HJ, Dankert J (**2000**). Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. **J Antimicrob Chemother** 46(2): 171-179. doi: 10.1093/jac/46.2.171.
354. Dovnik A, Golle A, Novak D, Arko D, Takac I (**2015**). Treatment of vulvovaginal candidiasis: a review of the literature. **Acta Dermatovenerol Alp Pannonica Adriat** 24(1): 5-7. doi: 10.15570/actaapa.2015.2.
355. Sekhavat L, Tabatabaii A, Tezerjani FZ (**2011**). Oral fluconazole 150 mg single dose versus intra-vaginal clotrimazole treatment of acute vulvovaginal candidiasis. **Journal of infection and public health** 4(4): 195-199. doi: 10.1016/j.jiph.2011.05.006.
356. Sobel JD, Kapernick PS, Zervos M, Reed BD, Hooton T, Soper D, Nyirjesy P, Heine MW, Willems J, Panzer H, Wittes H (**2001**). Treatment of complicated *Candida* vaginitis: comparison of single and sequential doses of fluconazole. **Am J Obstet Gynecol** 185(2): 363-369. doi: 10.1067/mob.2001.115116.
357. Sobel JD, Wiesenfeld HC, Martens M, Danna P, Hooton TM, Rompalo A, Sperling M, Livengood C, 3rd, Horowitz B, Von Thron J, Edwards L, Panzer H, Chu TC (**2004**). Maintenance

fluconazole therapy for recurrent vulvovaginal candidiasis. **N Engl J Med** 351(9): 876-883. doi: 10.1056/NEJMoa033114.
358. Fan S, Liu X, Wu C, Xu L, Li J (**2015**). Vaginal nystatin versus oral fluconazole for the treatment for recurrent vulvovaginal candidiasis. **Mycopathologia** 179(1-2): 95-101. doi: 10.1007/s11046-014-9827-4.
359. Phillips AJ (**2005**). Treatment of non-albicans *Candida* vaginitis with amphotericin B vaginal suppositories. **Am J Obstet Gynecol** 192(6): 2009-2012; discussion 2012-2003. doi: 10.1016/j.ajog.2005.03.034.
360. Denning DW (**2003**). Echinocandin antifungal drugs. **Lancet** 362(9390): 1142-1151. doi: 10.1016/S0140-6736(03)14472-8.
361. Sobel JD, Chaim W, Nagappan V, Leaman D (**2003**). Treatment of vaginitis caused by *Candida glabrata*: use of topical boric acid and flucytosine. **Am J Obstet Gynecol** 189(5): 1297-1300. doi: 10.1067/s0002-9378(03)00726-9.
362. Bondaryk M, Kurzatkowski W, Staniszewska M (**2013**). Antifungal agents commonly used in the superficial and mucosal candidiasis treatment: mode of action and resistance development. **Postepy Dermatol Alergol** 30(5): 293-301. doi: 10.5114/pdia.2013.38358.
363. Matsubara VH, Bandara HM, Mayer MP, Samaranayake LP (**2016**). Probiotics as Antifungals in Mucosal Candidiasis. **Clin Infect Dis** 62(9): 1143-1153. doi: 10.1093/cid/ciw038.
364. Rodrigues CF, Rodrigues ME, Henriques MCR (**2019**). Promising Alternative Therapeutics for Oral Candidiasis. **Curr Med Chem** 26(14): 2515-2528. doi: 10.2174/0929867325666180601102333.
365. Jeżewska-Frąckowiak J, Seroczyńska K, Banaszczyk J, Jedrzejczak G, Żylicz-Stachula A, Skowron PM (**2018**). The promises and risks of probiotic *Bacillus* species. **Acta Biochimica Polonica** 65(4): 509-519. doi: 10.18388/abp.2018_2652.
366. Pais P, Almeida V, Yilmaz M, Teixeira MC (**2020**). *Saccharomyces boulardii*: What Makes It Tick as Successful Probiotic? **J Fungi (Basel)** 6(2). doi: 10.3390/jof6020078.
367. Wescombe PA, Heng NC, Burton JP, Chilcott CN, Tagg JR (**2009**). Streptococcal bacteriocins and the case for *Streptococcus salivarius* as model oral probiotics. **Future Microbiol** 4(7): 819-835. doi: 10.2217/fmb.09.61.
368. Xie HY, Feng D, Wei DM, Mei L, Chen H, Wang X, Fang F (**2017**). Probiotics for vulvovaginal candidiasis in non-pregnant women. **Cochrane Database Syst Rev** 11(11): CD010496. doi: 10.1002/14651858.CD010496.pub2.
369. Kovachev SM, Vatcheva-Dobrevska RS (**2015**). Local Probiotic Therapy for Vaginal *Candida albicans* Infections. **Probiotics Antimicrob Proteins** 7(1): 38-44. doi: 10.1007/s12602-014-9176-0.
370. Ang XY, Mageswaran UM, Chung YLF, Lee BK, Azhar SNA, Roslan NS, Saufian IFB, Mustaffa NS, Kalam EM, Ibrahim AF, Abdul Wahid N, Deris ZZ, Oon CE, Adnan WFW, Sany S, Liong MT (**2022**). Probiotics Reduce Vaginal Candidiasis in Pregnant Women via Modulating Abundance of *Candida* and *Lactobacillus* in Vaginal and Cervicovaginal Regions. **Microorganisms** 10(2). doi: 10.3390/microorganisms10020285.
371. Palacios S, Espadaler J, Fernandez-Moya JM, Prieto C, Salas N (**2016**). Is it possible to prevent recurrent vulvovaginitis? The role of *Lactobacillus plantarum* I1001 (CECT7504). **Eur J Clin Microbiol Infect Dis** 35(10): 1701-1708. doi: 10.1007/s10096-016-2715-8.
372. Mollazadeh-Narestan Z, Yavarikia P, Homayouni-Rad A, Samadi Kafil H, Mohammad-Alizadeh-Charandabi S, Gholizadeh P, Mirghafourvand M (**2022**). Comparing the Effect of Probiotic and Fluconazole on Treatment and Recurrence of Vulvovaginal Candidiasis: a Triple-Blinded Randomized Controlled Trial. **Probiotics Antimicrob Proteins**: 1-11. doi: 10.1007/s12602-022-09997-3.
373. Pendharkar S, Brandsborg E, Hammarstrom L, Marcotte H, Larsson PG (**2015**). Vaginal colonisation by probiotic lactobacilli and clinical outcome in women conventionally treated for bacterial vaginosis and yeast infection. **BMC Infect Dis** 15(255. doi: 10.1186/s12879-015-0971-3.
374. Davar R, Nokhostin F, Eftekhar M, Sekhavat L, Bashiri Zadeh M, Shamsi F (**2016**). Comparing the Recurrence of Vulvovaginal Candidiasis in Patients Undergoing Prophylactic Treatment with Probiotic and Placebo During the 6 Months. **Probiotics Antimicrob Proteins** 8(3): 130-133. doi: 10.1007/s12602-016-9218-x.
375. Vicariotto F, Del Piano M, Mogna L, Mogna G (**2012**). Effectiveness of the association of 2 probiotic strains formulated in a slow release vaginal product, in women affected by vulvovaginal candidiasis: a pilot study. **J Clin Gastroenterol** 46 Suppl(S73-80. doi: 10.1097/MCG.0b013e3182684d71.

376. Murina F, Graziottin A, Vicariotto F, De Seta F (**2014**). Can *Lactobacillus fermentum* LF10 and *Lactobacillus acidophilus* LA02 in a slow-release vaginal product be useful for prevention of recurrent vulvovaginal candidiasis?: A clinical study. **J Clin Gastroenterol** 48 Suppl 1(S102-105. doi: 10.1097/MCG.0000000000000225.
377. Vladareanu R, Mihu D, Mitran M, Mehedintu C, Boiangiu A, Manolache M, Vladareanu S (**2018**). New evidence on oral *L. plantarum* P17630 product in women with history of recurrent vulvovaginal candidiasis (RVVC): a randomized double-blind placebo-controlled study. **Eur Rev Med Pharmacol Sci** 22(1): 262-267. doi: 10.26355/eurrev_201801_14128.
378. Kumar S, Bansal A, Chakrabarti A, Singhi S (**2013**). Evaluation of efficacy of probiotics in prevention of *Candida* colonization in a PICU-a randomized controlled trial. **Crit Care Med** 41(2): 565-572. doi: 10.1097/CCM.0b013e31826a409c.
379. Roy A, Chaudhuri J, Sarkar D, Ghosh P, Chakraborty S (**2014**). Role of Enteric Supplementation of Probiotics on Late-onset Sepsis by *Candida* species in Preterm Low Birth Weight Neonates: A Randomized, Double Blind, Placebo-controlled Trial. **N Am J Med Sci** 6(1): 50-57. doi: 10.4103/1947-2714.125870.
380. Severance EG, Gressitt KL, Stallings CR, Katsafanas E, Schweinfurth LA, Savage CLG, Adamos MB, Sweeney KM, Origoni AE, Khushalani S, Dickerson FB, Yolken RH (**2017**). Probiotic normalization of *Candida albicans* in schizophrenia: A randomized, placebo-controlled, longitudinal pilot study. **Brain Behav Immun** 62(41-45. doi: 10.1016/j.bbi.2016.11.019.
381. Manzoni P, Mostert M, Leonessa ML, Priolo C, Farina D, Monetti C, Latino MA, Gomirato G (**2006**). Oral supplementation with *Lactobacillus casei* subspecies rhamnosus prevents enteric colonization by *Candida* species in preterm neonates: a randomized study. **Clin Infect Dis** 42(12): 1735-1742. doi: 10.1086/504324.
382. Oncel MY, Arayici S, Sari FN, Simsek GK, Yurttutan S, Erdeve O, Saygan S, Uras N, Oguz SS, Dilmen U (**2015**). Comparison of *Lactobacillus reuteri* and nystatin prophylaxis on *Candida* colonization and infection in very low birth weight infants. **J Matern Fetal Neonatal Med** 28(15): 1790-1794. doi: 10.3109/14767058.2014.968842.
383. Anjum N, Maqsood S, Masud T, Ahmad A, Sohail A, Momin A (**2014**). *Lactobacillus acidophilus*: characterization of the species and application in food production. **Crit Rev Food Sci Nutr** 54(9): 1241-1251. doi: 10.1080/10408398.2011.621169.
384. Naghmouchi K, Belguesmia Y, Bendali F, Spano G, Seal BS, Drider D (**2020**). *Lactobacillus fermentum*: a bacterial species with potential for food preservation and biomedical applications. **Crit Rev Food Sci Nutr** 60(20): 3387-3399. doi: 10.1080/10408398.2019.1688250.
385. Toscano M, De Grandi R, Pastorelli L, Vecchi M, Drago L (**2017**). A consumer's guide for probiotics: 10 golden rules for a correct use. **Dig Liver Dis** 49(11): 1177-1184. doi: 10.1016/j.dld.2017.07.011.
386. Binda S, Hill C, Johansen E, Obis D, Pot B, Sanders ME, Tremblay A, Ouwehand AC (**2020**). Criteria to Qualify Microorganisms as "Probiotic" in Foods and Dietary Supplements. **Front Microbiol** 11(1662. doi: 10.3389/fmicb.2020.01662.
387. Matsubara VH, Wang Y, Bandara H, Mayer MPA, Samaranayake LP (**2016**). Probiotic lactobacilli inhibit early stages of *Candida* albicans biofilm development by reducing their growth, cell adhesion, and filamentation. **Appl Microbiol Biotechnol** 100(14): 6415-6426. doi: 10.1007/s00253-016-7527-3.
388. Tan Y, Leonhard M, Moser D, Ma S, Schneider-Stickler B (**2018**). Inhibitory effect of probiotic lactobacilli supernatants on single and mixed non-albicans *Candida* species biofilm. **Arch Oral Biol** 85(40-45. doi: 10.1016/j.archoralbio.2017.10.002.
389. Matsuda Y, Cho O, Sugita T, Ogishima D, Takeda S (**2018**). Culture Supernatants of *Lactobacillus gasseri* and *L. crispatus* Inhibit *Candida albicans* Biofilm Formation and Adhesion to HeLa Cells. **Mycopathologia** 183(4): 691-700. doi: 10.1007/s11046-018-0259-4.
390. Parolin C, Marangoni A, Laghi L, Foschi C, Nahui Palomino RA, Calonghi N, Cevenini R, Vitali B (**2015**). Isolation of Vaginal Lactobacilli and Characterization of Anti-*Candida* Activity. **PLoS One** 10(6): e0131220. doi: 10.1371/journal.pone.0131220.
391. Ribeiro FC, de Barros PP, Rossoni RD, Junqueira JC, Jorge AO (**2017**). *Lactobacillus rhamnosus* inhibits *Candida albicans* virulence factors *in vitro* and modulates immune system in *Galleria mellonella*. **J Appl Microbiol** 122(1): 201-211. doi: 10.1111/jam.13324.
392. Strus M, Kucharska A, Kukla G, Brzychczy-Wloch M, Maresz K, Heczko PB (**2005**). The *in vitro* activity of vaginal Lactobacillus with probiotic properties against *Candida*. **Infect Dis Obstet Gynecol** 13(2): 69-75. doi: 10.1080/10647440400028136.
393. Jang SJ, Lee K, Kwon B, You HJ, Ko G (**2019**). Vaginal lactobacilli inhibit growth and hyphae formation of *Candida albicans*. **Scientific reports** 9(1): 8121. doi: 10.1038/s41598-019-44579-4.

394. Wang S, Wang Q, Yang E, Yan L, Li T, Zhuang H (**2017**). Antimicrobial Compounds Produced by Vaginal *Lactobacillus crispatus* Are Able to Strongly Inhibit *Candida albicans* Growth, Hyphal Formation and Regulate Virulence-related Gene Expressions. **Front Microbiol** 8(564. doi: 10.3389/fmicb.2017.00564.
395. MacAlpine J, Daniel-Ivad M, Liu Z, Yano J, Revie NM, Todd RT, Stogios PJ, Sanchez H, O'Meara TR, Tompkins TA, Savchenko A, Selmecki A, Veri AO, Andes DR, Fidel PL, Jr., Robbins N, Nodwell J, Whitesell L, Cowen LE (**2021**). A small molecule produced by *Lactobacillus* species blocks *Candida albicans* filamentation by inhibiting a DYRK1-family kinase. **Nat Commun** 12(1): 6151. doi: 10.1038/s41467-021-26390-w.
396. Goyard S, Knechtle P, Chauvel M, Mallet A, Prevost MC, Proux C, Coppee JY, Schwarz P, Dromer F, Park H, Filler SG, Janbon G, d'Enfert C (**2008**). The Yak1 kinase is involved in the initiation and maintenance of hyphal growth in *Candida albicans*. **Mol Biol Cell** 19(5): 2251-2266. doi: 10.1091/mbc.e07-09-0960.
397. Parolin C, Croatti V, Laghi L, Giordani B, Tondi MR, De Gregorio PR, Foschi C, Vitali B (**2021**). *Lactobacillus* Biofilms Influence Anti-Candida Activity. **Front Microbiol** 12(750368. doi: 10.3389/fmicb.2021.750368.
398. Alonso-Roman R, Last A, Mirhakkak MH, Sprague JL, Möller L, Großmann P, Graf K, Gratz R, Mogavero S, Vylkova S (**2022**). *Lactobacillus rhamnosus* colonisation antagonizes *Candida albicans* by forcing metabolic adaptations that compromise pathogenicity. **Nature Communications** 13(1): 3192. doi: 10.1038/s41467-022-30661-5.
399. Lipinska-Zubrycka L, Klewicki R, Sojka M, Bonikowski R, Milczarek A, Klewicka E (**2020**). Anticandidal activity of *Lactobacillus* spp. in the presence of galactosyl polyols. **Microbiol Res** 240(126540. doi: 10.1016/j.micres.2020.126540.
400. Cortes-Zavaleta O, Lopez-Malo A, Hernandez-Mendoza A, Garcia HS (**2014**). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. **Int J Food Microbiol** 173(30-35. doi: 10.1016/j.ijfoodmicro.2013.12.016.
401. Niku-Paavola ML, Laitila A, Mattila-Sandholm T, Haikara A (**1999**). New types of antimicrobial compounds produced by *Lactobacillus plantarum*. **J Appl Microbiol** 86(1): 29-35. doi: 10.1046/j.1365-2672.1999.00632.x.
402. Nieminen MT, Novak-Frazer L, Rautemaa V, Rajendran R, Sorsa T, Ramage G, Bowyer P, Rautemaa R (**2014**). A novel antifungal is active against *Candida albicans* biofilms and inhibits mutagenic acetaldehyde production *in vitro*. **PLoS One** 9(5): e97864. doi: 10.1371/journal.pone.0097864.
403. Sakko M, Moore C, Novak-Frazer L, Rautemaa V, Sorsa T, Hietala P, Jarvinen A, Bowyer P, Tjaderhane L, Rautemaa R (**2014**). 2-hydroxyisocaproic acid is fungicidal for *Candida* and *Aspergillus* species. **Mycoses** 57(4): 214-221. doi: 10.1111/myc.12145.
404. Axel C, Brosnan B, Zannini E, Peyer LC, Furey A, Coffey A, Arendt EK (**2016**). Antifungal activities of three different *Lactobacillus* species and their production of antifungal carboxylic acids in wheat sourdough. **Appl Microbiol Biotechnol** 100(4): 1701-1711. doi: 10.1007/s00253-015-7051-x.
405. Radivojevic J, Skaro S, Senerovic L, Vasiljevic B, Guzik M, Kenny ST, Maslak V, Nikodinovic-Runic J, O'Connor KE (**2016**). Polyhydroxyalkanoate-based 3-hydroxyoctanoic acid and its derivatives as a platform of bioactive compounds. **Appl Microbiol Biotechnol** 100(1): 161-172. doi: 10.1007/s00253-015-6984-4.
406. Tachedjian G, Aldunate M, Bradshaw CS, Cone RA (**2017**). The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. **Res Microbiol** 168(9-10): 782-792. doi: 10.1016/j.resmic.2017.04.001.
407. Cherpes TL, Hillier SL, Meyn LA, Busch JL, Krohn MA (**2008**). A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. **Sex Transm Dis** 35(1): 78-83. doi: 10.1097/OLQ.0b013e318156a5d0.
408. Stoyancheva G, Marzotto M, Dellaglio F, Torriani S (**2014**). Bacteriocin production and gene sequencing analysis from vaginal *Lactobacillus* strains. **Arch Microbiol** 196(9): 645-653. doi: 10.1007/s00203-014-1003-1.
409. Satpute SK, Kulkarni GR, Banpurkar AG, Banat IM, Mone NS, Patil RH, Cameotra SS (**2016**). Biosurfactant/s from Lactobacilli species: Properties, challenges and potential biomedical applications. **J Basic Microbiol** 56(11): 1140-1158. doi: 10.1002/jobm.201600143.
410. Li S, Huang R, Shah NP, Tao X, Xiong Y, Wei H (**2014**). Antioxidant and antibacterial activities of exopolysaccharides from *Bifidobacterium bifidum* WBIN03 and *Lactobacillus plantarum* R315. **J Dairy Sci** 97(12): 7334-7343. doi: 10.3168/jds.2014-7912.

411. Aldunate M, Srbinovski D, Hearps AC, Latham CF, Ramsland PA, Gugasyan R, Cone RA, Tachedjian G (**2015**). Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. **Front Physiol** 6(164. doi: 10.3389/fphys.2015.00164.
412. Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA (**1999**). Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. **Infect Immun** 67(10): 5170-5175. doi: 10.1128/IAI.67.10.5170-5175.1999.
413. Boskey ER, Cone RA, Whaley KJ, Moench TR (**2001**). Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. **Hum Reprod** 16(9): 1809-1813. doi: 10.1093/humrep/16.9.1809.
414. CATO EP, Moore W, JOHNSON JL (**1983**). Synonymy of strains of "*Lactobacillus acidophilus*" group A2 (Johnson et al. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. **International Journal of Systematic and Evolutionary Microbiology** 33(2): 426-428. doi: 10.1099/00207713-33-2-426.
415. Falsen E, Pascual C, Sjoden B, Ohlen M, Collins MD (**1999**). Phenotypic and phylogenetic characterization of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp. nov. **Int J Syst Bacteriol** 49 Pt 1(217-221. doi: 10.1099/00207713-49-1-217.
416. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ (**2013**). Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections. **mBio** 4(4). doi: 10.1128/mBio.00460-13.
417. Baldewijns S, Sillen M, Palmans I, Vandecruys P, Van Dijck P, Demuyser L (**2021**). The Role of Fatty Acid Metabolites in Vaginal Health and Disease: Application to Candidiasis. **Front Microbiol** 12(705779. doi: 10.3389/fmicb.2021.705779.
418. Pivarcsi A, Nagy I, Koreck A, Kis K, Kenderessy-Szabo A, Szell M, Dobozy A, Kemeny L (**2005**). Microbial compounds induce the expression of pro-inflammatory cytokines, chemokines and human beta-defensin-2 in vaginal epithelial cells. **Microbes Infect** 7(9-10): 1117-1127. doi: 10.1016/j.micinf.2005.03.016.
419. Kumar R, Kaur M, Garsa AK, Shrivastava B, Reddy V, Tyagi A (**2015**). Natural and cultured buttermilk. **Fermented milk and dairy products**: 203-225. doi: 10.1201/b18987-14.
420. Xu D, Ding W, Ke W, Li F, Zhang P, Guo X (**2018**). Modulation of Metabolome and Bacterial Community in Whole Crop Corn Silage by Inoculating Homofermentative *Lactobacillus plantarum* and Heterofermentative *Lactobacillus buchneri*. **Front Microbiol** 9(3299. doi: 10.3389/fmicb.2018.03299.
421. Coudray MS, Madhivanan P (**2020**). Bacterial vaginosis-A brief synopsis of the literature. **Eur J Obstet Gynecol Reprod Biol** 245(143-148. doi: 10.1016/j.ejogrb.2019.12.035.
422. Gross S, Robbins EI (**2000**). Acidophilic and acid-tolerant fungi and yeasts. **Hydrobiologia** 433(1-3): 91-109. doi: 10.1023/a:1004014603333.
423. Booth I, Stratford M (**2003**). Acidulants and low pH. **Food preservatives**: 25-47. doi: 10.1007/978-0-387-30042-9_3.
424. Carmelo V, Bogaerts P, Sa-Correia I (**1996**). Activity of plasma membrane H+-ATPase and expression of PMA1 and PMA2 genes in *Saccharomyces cerevisiae* cells grown at optimal and low pH. **Arch Microbiol** 166(5): 315-320. doi: 10.1007/s002030050389.
425. Mira NP, Teixeira MC, Sa-Correia I (**2010**). Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. **OMICS** 14(5): 525-540. doi: 10.1089/omi.2010.0072.
426. Stevens S, Hofmeyr J-HS (**1993**). Effects of ethanol, octanoic and decanoic acids on fermentation and the passive influx of protons through the plasma membrane of *Saccharomyces cerevisiae*. **Applied Microbiology and Biotechnology** 38(656-663. doi: 10.1007/bf00182806.
427. Teixeira MC, Santos PM, Fernandes AR, Sa-Correia I (**2005**). A proteome analysis of the yeast response to the herbicide 2,4-dichlorophenoxyacetic acid. **Proteomics** 5(7): 1889-1901. doi: 10.1002/pmic.200401085.
428. Sá-Correia I, Salgueiro S, Viegas C, Novais J (**1989**). Leakage induced by ethanol, octanoic and decanoic acids in *Saccharomyces cerevisiae* (1989) Yeast, 5, pp. **S123-S127**. doi.
429. Owen DH, Katz DF (**1999**). A vaginal fluid simulant. **Contraception** 59(2): 91-95. doi: 10.1016/s0010-7824(99)00010-4.
430. Lourenco A, Pedro NA, Salazar SB, Mira NP (**2018**). Effect of Acetic Acid and Lactic Acid at Low pH in Growth and Azole Resistance of *Candida albicans* and *Candida glabrata*. **Front Microbiol** 9(3265. doi: 10.3389/fmicb.2018.03265.

431. Koziolek M, Grimm M, Becker D, Iordanov V, Zou H, Shimizu J, Wanke C, Garbacz G, Weitschies W (**2015**). Investigation of pH and Temperature Profiles in the GI Tract of Fasted Human Subjects Using the Intellicap((R)) System. **J Pharm Sci** 104(9): 2855-2863. doi: 10.1002/jps.24274.
432. Fallingborg J (**1999**). Intraluminal pH of the human gastrointestinal tract. **Danish medical bulletin** 46(3): 183-196. doi: PMID: 10421978.
433. Ueno K, Matsumoto Y, Uno J, Sasamoto K, Sekimizu K, Kinjo Y, Chibana H (**2011**). Intestinal resident yeast *Candida glabrata* requires Cyb2p-mediated lactate assimilation to adapt in mouse intestine. **PLoS One** 6(9): e24759. doi: 10.1371/journal.pone.0024759.
434. Ene IV, Cheng SC, Netea MG, Brown AJ (**2013**). Growth of *Candida albicans* cells on the physiologically relevant carbon source lactate affects their recognition and phagocytosis by immune cells. **Infect Immun** 81(1): 238-248. doi: 10.1128/IAI.01092-12.
435. Noble SM, Gianetti BA, Witchley JN (**2017**). *Candida albicans* cell-type switching and functional plasticity in the mammalian host. **Nat Rev Microbiol** 15(2): 96-108. doi: 10.1038/nrmicro.2016.157.
436. Maresh JJ, Giddings LA, Friedrich A, Loris EA, Panjikar S, Trout BL, Stockigt J, Peters B, O'Connor SE (**2008**). Strictosidine synthase: mechanism of a Pictet-Spengler catalyzing enzyme. **J Am Chem Soc** 130(2): 710-723. doi: 10.1021/ja077190z.
437. Szabo T, Volk B, Milen M (**2021**). Recent Advances in the Synthesis of beta-Carboline Alkaloids. **Molecules** 26(3). doi: 10.3390/molecules26030663.
438. Chen Q, Zhang S, Xie Y (**2018**). Characterization of a new microbial Pictet-Spenglerase NscbB affording the beta-carboline skeletons from *Nocardiopsis synnemataformans* DSM 44143. **J Biotechnol** 281(137-143. doi: 10.1016/j.jbiotec.2018.07.007.
439. Chen Q, Ji C, Song Y, Huang H, Ma J, Tian X, Ju J (**2013**). Discovery of McbB, an enzyme catalyzing the beta-carboline skeleton construction in the marinacarboline biosynthetic pathway. **Angew Chem Int Ed Engl** 52(38): 9980-9984. doi: 10.1002/anie.201303449.
440. Mori T, Hoshino S, Sahashi S, Wakimoto T, Matsui T, Morita H, Abe I (**2015**). Structural Basis for beta-Carboline Alkaloid Production by the Microbial Homodimeric Enzyme McbB. **Chem Biol** 22(7): 898-906. doi: 10.1016/j.chembiol.2015.06.006.
441. Ueda S, Ikeda H, Namba T, Ikejiri Y, Nishimoto Y, Arai M, Nihira T, Kitani S (**2019**). Identification of biosynthetic genes for the beta-carboline alkaloid kitasetaline and production of the fluorinated derivatives by heterologous expression. **J Ind Microbiol Biotechnol** 46(5): 739-750. doi: 10.1007/s10295-019-02151-z.
442. Fuochi V, Cardile V, Petronio Petronio G, Furneri PM (**2019**). Biological properties and production of bacteriocins-like-inhibitory substances by *Lactobacillus* sp. strains from human vagina. **Journal of applied microbiology** 126(5): 1541-1550. doi: 10.1111/jam.14164.
443. Aroutcheva AA, Simoes JA, Faro S (**2001**). Antimicrobial protein produced by vaginal *Lactobacillus acidophilus* that inhibits *Gardnerella vaginalis*. **Infect Dis Obstet Gynecol** 9(1): 33-39. doi: 10.1155/S1064744901000060.
444. Turovskiy Y, Ludescher RD, Aroutcheva AA, Faro S, Chikindas ML (**2009**). Lactocin 160, a Bacteriocin Produced by Vaginal *Lactobacillus rhamnosus*, Targets Cytoplasmic Membranes of the Vaginal Pathogen, *Gardnerella vaginalis*. **Probiotics Antimicrob Proteins** 1(1): 67-74. doi: 10.1007/s12602-008-9003-6.
445. Mokoena MP (**2017**). Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. **Molecules** 22(8). doi: 10.3390/molecules22081255.
446. Kumariya R, Garsa AK, Rajput YS, Sood SK, Akhtar N, Patel S (**2019**). Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. **Microb Pathog** 128(171-177. doi: 10.1016/j.micpath.2019.01.002.
447. Negash AW, Tsehai BA (**2020**). Current Applications of Bacteriocin. **Int J Microbiol** 2020(4374891. doi: 10.1155/2020/4374891.
448. Alvarez-Sieiro P, Montalban-Lopez M, Mu D, Kuipers OP (**2016**). Bacteriocins of lactic acid bacteria: extending the family. **Appl Microbiol Biotechnol** 100(7): 2939-2951. doi: 10.1007/s00253-016-7343-9.
449. Kaur S, Kaur S (**2015**). Bacteriocins as Potential Anticancer Agents. **Front Pharmacol** 6(272. doi: 10.3389/fphar.2015.00272.
450. Vasilchenko AS, Valyshev AV (**2019**). Pore-forming bacteriocins: structural-functional relationships. **Arch Microbiol** 201(2): 147-154. doi: 10.1007/s00203-018-1610-3.
451. Hechard Y, Sahl HG (**2002**). Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. **Biochimie** 84(5-6): 545-557. doi: 10.1016/s0300-9084(02)01417-7.

452. Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, Sahl HG (**2001**). Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. **J Biol Chem** 276(3): 1772-1779. doi: 10.1074/jbc.M006770200.
453. Perez RH, Perez MTM, Elegado FB (**2015**). Bacteriocins from Lactic Acid Bacteria: A Review of Biosynthesis, Mode of Action, Fermentative Production, Uses, and Prospects. doi: 10.18191/2015-08-2-027.
454. Morgan SM, O'Connor P M, Cotter PD, Ross RP, Hill C (**2005**). Sequential actions of the two component peptides of the lantibiotic lacticin 3147 explain its antimicrobial activity at nanomolar concentrations. **Antimicrob Agents Chemother** 49(7): 2606-2611. doi: 10.1128/AAC.49.7.2606-2611.2005.
455. Wiedemann I, Bottiger T, Bonelli RR, Wiese A, Hagge SO, Gutsmann T, Seydel U, Deegan L, Hill C, Ross P, Sahl HG (**2006**). The mode of action of the lantibiotic lacticin 3147--a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. **Mol Microbiol** 61(2): 285-296. doi: 10.1111/j.1365-2958.2006.05223.x.
456. Moll G, Hildeng-Hauge H, Nissen-Meyer J, Nes IF, Konings WN, Driessen AJ (**1998**). Mechanistic properties of the two-component bacteriocin lactococcin G. **J Bacteriol** 180(1): 96-99. doi: 10.1128/JB.180.1.96-99.1998.
457. Nissen-Meyer J, Oppegard C, Rogne P, Haugen HS, Kristiansen PE (**2010**). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. **Probiotics Antimicrob Proteins** 2(1): 52-60. doi: 10.1007/s12602-009-9021-z.
458. Dover S, Aroutcheva A, Faro S, Chikindas M (**2008**). Natural antimicrobials and their role in vaginal health: a short review. **International journal of probiotics & prebiotics** 3(4): 219. doi: PMID: 20657710.
459. Li J, Aroutcheva AA, Faro S, Chikindas ML (**2005**). Mode of action of lactocin 160, a bacteriocin from vaginal *Lactobacillus rhamnosus*. **Infect Dis Obstet Gynecol** 13(3): 135-140. doi: 10.1080/10647440500148156.
460. Todorov SD, Danova ST, Van Reenen CA, Meincken M, Dinkova G, Ivanova IV, Dicks LM (**2006**). Characterization of bacteriocin HV219, produced by *Lactococcus lactis* subsp. lactis HV219 isolated from human vaginal secretions. **J Basic Microbiol** 46(3): 226-238. doi: 10.1002/jobm.200510037.
461. Kaur B, Balgir PP, Mittu B, Kumar B, Garg N (**2013**). Biomedical applications of fermenticin HV6b isolated from *Lactobacillus fermentum* HV6b MTCC10770. **Biomed Res Int** 2013(168438. doi: 10.1155/2013/168438.
462. Sutyak KE, Anderson RA, Dover SE, Feathergill KA, Aroutcheva AA, Faro S, Chikindas ML (**2008**). Spermicidal activity of the safe natural antimicrobial peptide subtilosin. **Infect Dis Obstet Gynecol** 2008(540758. doi: 10.1155/2008/540758.
463. Borges S, Barbosa J, Silva J, Teixeira P (**2013**). Evaluation of characteristics of *Pediococcus* spp. to be used as a vaginal probiotic. **J Appl Microbiol** 115(2): 527-538. doi: 10.1111/jam.12232.
464. Dezwaan DC, Mequio MJ, Littell JS, Allen JP, Rossbach S, Pybus V (**2007**). Purification and characterization of enterocin 62-6, a two-peptide bacteriocin produced by a vaginal strain of *Enterococcus faecium*: Potential significance in bacterial vaginosis. **Microb Ecol Health Dis** 19(4): 241-250. doi: 10.1080/08910600701538240.
465. Gaspar C, Donders GG, Palmeira-de-Oliveira R, Queiroz JA, Tomaz C, Martinez-de-Oliveira J, Palmeira-de-Oliveira A (**2018**). Bacteriocin production of the probiotic *Lactobacillus acidophilus* KS400. **AMB Express** 8(1): 153. doi: 10.1186/s13568-018-0679-z.
466. Maldonado-Barragan A, Caballero-Guerrero B, Martin V, Ruiz-Barba JL, Rodriguez JM (**2016**). Purification and genetic characterization of gassericin E, a novel co-culture inducible bacteriocin from *Lactobacillus gasseri* EV1461 isolated from the vagina of a healthy woman. **BMC Microbiol** 16(37. doi: 10.1186/s12866-016-0663-1.
467. Okkers DJ, Dicks LM, Silvester M, Joubert JJ, Odendaal HJ (**1999**). Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with a fungistatic effect on *Candida albicans*. **J Appl Microbiol** 87(5): 726-734. doi: 10.1046/j.1365-2672.1999.00918.x.
468. Sharma A, Srivastava S (**2014**). Anti-Candida activity of two-peptide bacteriocins, plantaricins (Pln E/F and J/K) and their mode of action. **Fungal Biol** 118(2): 264-275. doi: 10.1016/j.funbio.2013.12.006.
469. Graham CE, Cruz MR, Garsin DA, Lorenz MC (**2017**). *Enterococcus faecalis* bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of *Candida albicans*. **Proc Natl Acad Sci U S A** 114(17): 4507-4512. doi: 10.1073/pnas.1620432114.

470. Belguesmia Y, Choiset Y, Rabesona H, Baudy-Floc'h M, Le Blay G, Haertle T, Chobert JM (**2013**). Antifungal properties of durancins isolated from *Enterococcus durans* A5-11 and of its synthetic fragments. **Lett Appl Microbiol** 56(4): 237-244. doi: 10.1111/lam.12037.
471. Mohsin ZA (**2021**). Antagonistic activity of bacteriocin-producing *Lactobacillus* against *Candida* spp. **Iraqi Journal of Science**: 2153-2162. doi: 10.24996/ijs.2021.62.7.4.
472. Hefzy EM, Khalil MAF, Amin AAI, Ashour HM, Abdelaliem YF (**2021**). Bacteriocin-Like Inhibitory Substances from Probiotics as Therapeutic Agents for *Candida* Vulvovaginitis. **Antibiotics (Basel)** 10(3). doi: 10.3390/antibiotics10030306.
473. Zhou X, Yang B, Stanton C, Ross RP, Zhao J, Zhang H, Chen W (**2020**). Comparative analysis of *Lactobacillus gasseri* from Chinese subjects reveals a new species-level taxa. **BMC Genomics** 21(1): 119. doi: 10.1186/s12864-020-6527-y.
474. Majhenic AC, Venema K, Allison GE, Matijasic BB, Rogelj I, Klaenhammer TR (**2004**). DNA analysis of the genes encoding acidocin LF221 A and acidocin LF221 B, two bacteriocins produced by *Lactobacillus gasseri* LF221. **Appl Microbiol Biotechnol** 63(6): 705-714. doi: 10.1007/s00253-003-1424-2.
475. Pandey N, Malik RK, Kaushik JK, Singroha G (**2013**). Gassericin A: a circular bacteriocin produced by lactic acid bacteria *Lactobacillus gasseri*. **World J Microbiol Biotechnol** 29(11): 1977-1987. doi: 10.1007/s11274-013-1368-3.
476. Soltani S, Couture F, Boutin Y, Ben Said L, Cashman-Kadri S, Subirade M, Biron E, Fliss I (**2021**). *In vitro* investigation of gastrointestinal stability and toxicity of 3-hyrdoxypropionaldehyde (reuterin) produced by *Lactobacillus reuteri*. **Toxicol Rep** 8(740-746. doi: 10.1016/j.toxrep.2021.03.025.
477. Chen SY, Yang RS, Ci BQ, Xin WG, Zhang QL, Lin LB, Wang F (**2023**). A novel bacteriocin against multiple foodborne pathogens from *Lacticaseibacillus rhamnosus* isolated from juice ferments: ATF perfusion-based preparation of viable cells, characterization, antibacterial and antibiofilm activity. **Curr Res Food Sci** 6(100484. doi: 10.1016/j.crfs.2023.100484.
478. Dimitrijevic R, Stojanovic M, Zivkovic I, Petersen A, Jankov RM, Dimitrijevic L, Gavrovic-Jankulovic M (**2009**). The identification of a low molecular mass bacteriocin, rhamnosin A, produced by *Lactobacillus rhamnosus* strain 68. **J Appl Microbiol** 107(6): 2108-2115. doi: 10.1111/j.1365-2672.2009.04539.x.
479. Muriana PM, Klaenhammer TR (**1991**). Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. **Appl Environ Microbiol** 57(1): 114-121. doi: 10.1128/aem.57.1.114-121.1991.
480. Barefoot SF, Klaenhammer TR (**1984**). Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. **Antimicrob Agents Chemother** 26(3): 328-334. doi: 10.1128/AAC.26.3.328.
481. Li L, Zhang J, Zhou L, Shi H, Mai H, Su J, Ma X, Zhong J (**2023**). The First Lanthipeptide from *Lactobacillus iners*, Inecin L, Exerts High Antimicrobial Activity against Human Vaginal Pathogens. **Appl Environ Microbiol** 89(3): e0212322. doi: 10.1128/aem.02123-22.
482. Stoyancheva G (**2020**). Study of helveticin gene in *Lactobacillus crispatus* strains and evaluation of its use as a phylogenetic marker. **Arch Microbiol** 202(1): 205-208. doi: 10.1007/s00203-019-01711-2.
483. Forde BM, Neville BA, O'Donnell MM, Riboulet-Bisson E, Claesson MJ, Coghlan A, Ross RP, O'Toole PW (**2011**). Genome sequences and comparative genomics of two *Lactobacillus ruminis* strains from the bovine and human intestinal tracts. **Microb Cell Fact** 10 Suppl 1(Suppl 1): S13. doi: 10.1186/1475-2859-10-S1-S13.
484. Abdulhussain Kareem R, Razavi SH (**2020**). Plantaricin bacteriocins: As safe alternative antimicrobial peptides in food preservation—A review. **Journal of Food Safety** 40(1): e12735. doi: 10.1111/jfs.12735.
485. Messaoudi S, Manai M, Kergourlay G, Prevost H, Connil N, Chobert JM, Dousset X (**2013**). *Lactobacillus salivarius*: bacteriocin and probiotic activity. **Food Microbiol** 36(2): 296-304. doi: 10.1016/j.fm.2013.05.010.
486. Hillier SL, Krohn MA, Rabe LK, Klebanoff SJ, Eschenbach DA (**1993**). The normal vaginal flora, H2O2-producing lactobacilli, and bacterial vaginosis in pregnant women. **Clin Infect Dis** 16 Suppl 4(S273-281. doi: 10.1093/clinids/16.supplement_4.s273.
487. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, Holmes KK (**1989**). Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. **J Clin Microbiol** 27(2): 251-256. doi: 10.1128/jcm.27.2.251-256.1989.

488. Mijač VD, Đukić SV, Opavski NZ, Đukić MK, Ranin LT (**2006**). Hydrogen peroxide producing lactobacilli in women with vaginal infections. **European Journal of Obstetrics & Gynecology and Reproductive Biology** 129(1): 69-76. doi: 10.1016/j.ejogrb.2005.11.036.
489. O'Hanlon DE, Lanier BR, Moench TR, Cone RA (**2010**). Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. **BMC Infect Dis** 10(120. doi: 10.1186/1471-2334-10-120.
490. Markande AR, Patel D, Varjani S (**2021**). A review on biosurfactants: properties, applications and current developments. **Bioresource Technology** 330(124963. doi: 10.1016/j.biortech.2021.124963.
491. Drakontis CE, Amin S (**2020**). Biosurfactants: Formulations, properties, and applications. **Current Opinion in Colloid & Interface Science** 48(77-90. doi: 10.1016/j.cocis.2020.03.013.
492. Eslami P, Hajfarajollah H, Bazsefidpar S (**2020**). Recent advancements in the production of rhamnolipid biosurfactants by *Pseudomonas aeruginosa*. **RSC Adv** 10(56): 34014-34032. doi: 10.1039/d0ra04953k.
493. Klausmann P, Lilge L, Aschern M, Hennemann K, Henkel M, Hausmann R, Morabbi Heravi K (**2021**). Influence of *B. subtilis* 3NA mutations in spo0A and abrB on surfactin production in *B. subtilis* 168. **Microb Cell Fact** 20(1): 188. doi: 10.1186/s12934-021-01679-z.
494. Velraeds MM, van de Belt-Gritter B, van der Mei HC, Reid G, Busscher HJ (**1998**). Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. **J Med Microbiol** 47(12): 1081-1085. doi: 10.1099/00222615-47-12-1081.
495. Ceresa C, Tessarolo F, Caola I, Nollo G, Cavallo M, Rinaldi M, Fracchia L (**2015**). Inhibition of *Candida albicans* adhesion on medical-grade silicone by a Lactobacillus-derived biosurfactant. **J Appl Microbiol** 118(5): 1116-1125. doi: 10.1111/jam.12760.
496. Fracchia L, Cavallo M, Allegrone G, Martinotti M (**2010**). A *Lactobacillus*-derived biosurfactant inhibits biofilm formation of human pathogenic *Candida albicans* biofilm producers. **Appl Microbiol Biotechnol** 2(827-837. doi.
497. Ch I M, Khanaqa H, H K F (**2010**). Purification and characterization of surlactin produced by *Lactobacillus acidophilus*. **Al-Anbar Medical Journal** 8(1): 34-39. doi.
498. De Gregorio PR, Parolin C, Abruzzo A, Luppi B, Protti M, Mercolini L, Silva JA, Giordani B, Marangoni A, Nader-Macias MEF, Vitali B (**2020**). Biosurfactant from vaginal *Lactobacillus crispatus* BC1 as a promising agent to interfere with *Candida* adhesion. **Microb Cell Fact** 19(1): 133. doi: 10.1186/s12934-020-01390-5.
499. Abruzzo A, Giordani B, Parolin C, De Gregorio PR, Foschi C, Cerchiara T, Bigucci F, Vitali B, Luppi B (**2021**). *Lactobacillus crispatus* BC1 Biosurfactant Delivered by Hyalurosomes: An Advanced Strategy to Counteract *Candida* Biofilm. **Antibiotics (Basel)** 10(1). doi: 10.3390/antibiotics10010033.
500. Nelson J, El-Gendy AO, Mansy MS, Ramadan MA, Aziz RK (**2020**). The biosurfactants iturin, lichenysin and surfactin, from vaginally isolated lactobacilli, prevent biofilm formation by pathogenic *Candida*. **FEMS Microbiol Lett** 367(15). doi: 10.1093/femsle/fnaa126.
501. Velraeds MM, van der Mei HC, Reid G, Busscher HJ (**1996**). Physicochemical and biochemical characterization of biosurfactants released by *Lactobacillus* strains. **Colloids and Surfaces B: Biointerfaces** 8(1-2): 51-61. doi: 10.1016/s0927-7765(96)01297-0.
502. Velraeds MM, van der Mei HC, Reid G, Busscher HJ (**1997**). Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* to solid substrata by an adsorbed biosurfactant layer from *Lactobacillus acidophilus*. **Urology** 49(5): 790-794. doi: 10.1016/S0090-4295(97)00065-4.
503. Rajoka MSR, Wu Y, Mehwish HM, Bansal M, Zhao L (**2020**). *Lactobacillus* exopolysaccharides: New perspectives on engineering strategies, physiochemical functions, and immunomodulatory effects on host health. **Trends in Food Science & Technology** 103(36-48. doi: 10.1016/j.tifs.2020.06.003.
504. Zeidan AA, Poulsen VK, Janzen T, Buldo P, Derkx PMF, Oregaard G, Neves AR (**2017**). Polysaccharide production by lactic acid bacteria: from genes to industrial applications. **FEMS Microbiol Rev** 41(Supp_1): S168-S200. doi: 10.1093/femsre/fux017.
505. Ojala T, Kankainen M, Castro J, Cerca N, Edelman S, Westerlund-Wikstrom B, Paulin L, Holm L, Auvinen P (**2014**). Comparative genomics of *Lactobacillus crispatus* suggests novel mechanisms for the competitive exclusion of *Gardnerella vaginalis*. **BMC Genomics** 15(1070. doi: 10.1186/1471-2164-15-1070.
506. Abdalla AK, Ayyash MM, Olaimat AN, Osaili TM, Al-Nabulsi AA, Shah NP, Holley R (**2021**). Exopolysaccharides as Antimicrobial Agents: Mechanism and Spectrum of Activity. **Front Microbiol** 12(664395. doi: 10.3389/fmicb.2021.664395.

507. Abouloifa H, Rokni Y, Bellaouchi R, Ghabbour N, Karboune S, Brasca M, Ben Salah R, Chihib NE, Saalaoui E, Asehraou A (**2020**). Characterization of Probiotic Properties of Antifungal *Lactobacillus* Strains Isolated from Traditional Fermenting Green Olives. **Probiotics Antimicrob Proteins** 12(2): 683-696. doi: 10.1007/s12602-019-09543-8.
508. Allonsius CN, van den Broek MFL, De Boeck I, Kiekens S, Oerlemans EFM, Kiekens F, Foubert K, Vandenheuvel D, Cos P, Delputte P, Lebeer S (**2017**). Interplay between *Lactobacillus rhamnosus* GG and *Candida* and the involvement of exopolysaccharides. **Microb Biotechnol** 10(6): 1753-1763. doi: 10.1111/1751-7915.12799.
509. Abedin-Do A, Taherian-Esfahani Z, Ghafouri-Fard S, Ghafouri-Fard S, Motevaseli E (**2015**). Immunomodulatory effects of *Lactobacillus* strains: emphasis on their effects on cancer cells. **Immunotherapy** 7(12): 1307-1329. doi: 10.2217/imt.15.92.
510. Rizzo A, Fiorentino M, Buommino E, Donnarumma G, Losacco A, Bevilacqua N (**2015**). *Lactobacillus crispatus* mediates anti-inflammatory cytokine interleukin-10 induction in response to *Chlamydia trachomatis* infection *in vitro*. **Int J Med Microbiol** 305(8): 815-827. doi: 10.1016/j.ijmm.2015.07.005.
511. Rizzo A, Losacco A, Carratelli CR (**2013**). *Lactobacillus crispatus* modulates epithelial cell defense against *Candida albicans* through Toll-like receptors 2 and 4, interleukin 8 and human beta-defensins 2 and 3. **Immunol Lett** 156(1-2): 102-109. doi: 10.1016/j.imlet.2013.08.013.
512. Hearps AC, Tyssen D, Srbinovski D, Bayigga L, Diaz DJD, Aldunate M, Cone RA, Gugasyan R, Anderson DJ, Tachedjian G (**2017**). Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. **Mucosal Immunol** 10(6): 1480-1490. doi: 10.1038/mi.2017.27.
513. Mitchell C, Fredricks D, Agnew K, Hitti J (**2015**). Hydrogen Peroxide-Producing Lactobacilli Are Associated With Lower Levels of Vaginal Interleukin-1beta, Independent of Bacterial Vaginosis. **Sex Transm Dis** 42(7): 358-363. doi: 10.1097/OLQ.0000000000000298.
514. Borjian Boroujeni Z, Shamsaei S, Yarahmadi M, Getso MI, Salimi Khorashad A, Haghighi L, Raissi V, Zareei M, Saleh Mohammadzade A, Moqarabzadeh V, Soleimani A, Raeisi F, Mohseni M, Mohseni MS, Raiesi O (**2021**). Distribution of invasive fungal infections: Molecular epidemiology, etiology, clinical conditions, diagnosis and risk factors: A 3-year experience with 490 patients under intensive care. **Microb Pathog** 152(104616. doi: 10.1016/j.micpath.2020.104616.
515. Mishra AA, Koh AY (**2021**). The microbial and host factors that govern *Candida* gastrointestinal colonization and dissemination. **Curr Opin Microbiol** 63(29-35. doi: 10.1016/j.mib.2021.05.012.
516. Denning DW, Bromley MJ (**2015**). Infectious Disease. How to bolster the antifungal pipeline. **Science** 347(6229): 1414-1416. doi: 10.1126/science.aaa6097.
517. Denning DW, Kneale M, Sobel JD, Rautemaa-Richardson R (**2018**). Global burden of recurrent vulvovaginal candidiasis: a systematic review. **The Lancet infectious diseases** 18(11): e339-e347. doi: 10.1016/S1473-3099(18)30103-8.
518. Seed PC (**2014**). The human mycobiome. **Cold Spring Harb Perspect Med** 5(5): a019810. doi: 10.1101/cshperspect.a019810.
519. Huseyin CE, O'Toole PW, Cotter PD, Scanlan PD (**2017**). Forgotten fungi-the gut mycobiome in human health and disease. **FEMS Microbiol Rev** 41(4): 479-511. doi: 10.1093/femsre/fuw047.
520. Serrano MG, Parikh HI, Brooks JP, Edwards DJ, Arodz TJ, Edupuganti L, Huang B, Girerd PH, Bokhari YA, Bradley SP (**2019**). Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. **Nature medicine** 25(6): 1001-1011. doi: 10.1038/s41591-019-0465-8.
521. Auriemma RS, Scairati R, Del Vecchio G, Liccardi A, Verde N, Pirchio R, Pivonello R, Ercolini D, Colao A (**2021**). The Vaginal Microbiome: A Long Urogenital Colonization Throughout Woman Life. **Front Cell Infect Microbiol** 11(686167. doi: 10.3389/fcimb.2021.686167.
522. Bommana S, Richards G, Kama M, Kodimerla R, Jijakli K, Read TD, Dean D (**2022**). Metagenomic Shotgun Sequencing of Endocervical, Vaginal, and Rectal Samples among Fijian Women with and without *Chlamydia trachomatis* Reveals Disparate Microbial Populations and Function across Anatomic Sites: a Pilot Study. **Microbiol Spectr** 10(3): e0010522. doi: 10.1128/spectrum.00105-22.
523. Lennard K, Dabee S, Barnabas SL, Havyarimana E, Blakney A, Jaumdally SZ, Botha G, Mkhize NN, Bekker LG, Lewis DA, Gray G, Mulder N, Passmore JS, Jaspan HB (**2018**). Microbial Composition Predicts Genital Tract Inflammation and Persistent Bacterial Vaginosis in South African Adolescent Females. **Infect Immun** 86(1). doi: 10.1128/IAI.00410-17.

524. Norenhag J, Du J, Olovsson M, Verstraelen H, Engstrand L, Brusselaers N (**2020**). The vaginal microbiota, human papillomavirus and cervical dysplasia: a systematic review and network meta-analysis. **BJOG** 127(2): 171-180. doi: 10.1111/1471-0528.15854.
525. McClelland RS, Lingappa JR, Srinivasan S, Kinuthia J, John-Stewart GC, Jaoko W, Richardson BA, Yuhas K, Fiedler TL, Mandaliya KN (**2018**). Evaluation of the association between the concentrations of key vaginal bacteria and the increased risk of HIV acquisition in African women from five cohorts: a nested case-control study. **The Lancet infectious diseases** 18(5): 554-564. doi: 10.1016/S1473-3099(18)30058-6.
526. De Seta F, Lonnee-Hoffmann R, Campisciano G, Comar M, Verstraelen H, Vieira-Baptista P, Ventolini G, Lev-Sagie A (**2022**). The Vaginal Microbiome: III. The Vaginal Microbiome in Various Urogenital Disorders. **J Low Genit Tract Dis** 26(1): 85-92. doi: 10.1097/LGT.0000000000000645.
527. Ilkit M, Guzel AB (**2011**). The epidemiology, pathogenesis, and diagnosis of vulvovaginal candidosis: a mycological perspective. **Crit Rev Microbiol** 37(3): 250-261. doi: 10.3109/1040841X.2011.576332.
528. Seelig MS (**1966**). Mechanisms by which antibiotics increase the incidence and severity of candidiasis and alter the immunological defenses. **Bacteriol Rev** 30(2): 442-459. doi: 10.1128/br.30.2.442-459.1966.
529. Kang CH, Kim Y, Han SH, Kim JS, Paek NS, So JS (**2018**). *In vitro* probiotic properties of vaginal *Lactobacillus fermentum* MG901 and *Lactobacillus plantarum* MG989 against *Candida albicans*. **Eur J Obstet Gynecol Reprod Biol** 228(232-237. doi: 10.1016/j.ejogrb.2018.07.005.
530. van de Wijgert J, Verwijs MC (**2020**). Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs. **BJOG** 127(2): 287-299. doi: 10.1111/1471-0528.15870.
531. Andrade JC, Kumar S, Kumar A, Cernakova L, Rodrigues CF (**2022**). Application of probiotics in candidiasis management. **Crit Rev Food Sci Nutr** 62(30): 8249-8264. doi: 10.1080/10408398.2021.1926905.
532. Shenoy A, Gottlieb A (**2019**). Probiotics for oral and vulvovaginal candidiasis: A review. **Dermatol Ther** 32(4): e12970. doi: 10.1111/dth.12970.
533. Zeise KD, Woods RJ, Huffnagle GB (**2021**). Interplay between *Candida albicans* and Lactic Acid Bacteria in the Gastrointestinal Tract: Impact on Colonization Resistance, Microbial Carriage, Opportunistic Infection, and Host Immunity. **Clin Microbiol Rev** 34(4): e0032320. doi: 10.1128/CMR.00323-20.
534. Graf K, Last A, Gratz R, Allert S, Linde S, Westermann M, Groger M, Mosig AS, Gresnigt MS, Hube B (**2019**). Keeping *Candida* commensal: how lactobacilli antagonize pathogenicity of *Candida albicans* in an *in vitro* gut model. **Dis Model Mech** 12(9). doi: 10.1242/dmm.039719.
535. Zangl I, Pap IJ, Aspock C, Schuller C (**2019**). The role of *Lactobacillus* species in the control of *Candida* via biotrophic interactions. **Microb Cell** 7(1): 1-14. doi: 10.15698/mic2020.01.702.
536. O'Hanlon DE, Moench TR, Cone RA (**2011**). In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. **BMC Infect Dis** 11(200. doi: 10.1186/1471-2334-11-200.
537. Mendling W, Shazly MAE, Zhang L (**2022**). The Role of Lactic Acid in the Management of Bacterial Vaginosis: A Systematic Literature Review. **Future Pharmacology** 2(3): 198-213. doi: 10.3390/futurepharmacol2030014.
538. O'Hanlon DE, Come RA, Moench TR (**2019**). Vaginal pH measured *in vivo*: lactobacilli determine pH and lactic acid concentration. **BMC Microbiol** 19(1): 13. doi: 10.1186/s12866-019-1388-8.
539. Santos CMA, Pires MCV, Leao TL, Silva AKS, Miranda LS, Martins FS, Silva AM, Nicoli JR (**2018**). Anti-inflammatory effect of two *Lactobacillus* strains during infection with *Gardnerella vaginalis* and *Candida albicans* in a HeLa cell culture model. **Microbiology (Reading)** 164(3): 349-358. doi: 10.1099/mic.0.000608.
540. Cunha DV, Salazar SB, Lopes MM, Mira NP (**2017**). Mechanistic Insights Underlying Tolerance to Acetic Acid Stress in Vaginal *Candida glabrata* Clinical Isolates. **Front Microbiol** 8(259. doi: 10.3389/fmicb.2017.00259.
541. Biswas S, Keightley A, Biswas I (**2019**). Characterization of a stress tolerance-defective mutant of *Lactobacillus rhamnosus* LRB. **Mol Oral Microbiol** 34(4): 153-167. doi: 10.1111/omi.12262.
542. Bhatia SJ, Kochar N, Abraham P, Nair NG, Mehta AP (**1989**). *Lactobacillus acidophilus* inhibits growth of *Campylobacter pylori in vitro*. **J Clin Microbiol** 27(10): 2328-2330. doi: 10.1128/jcm.27.10.2328-2330.1989.

543. Pinto SN, Dias SA, Cruz AF, Mil-Homens D, Fernandes F, Valle J, Andreu D, Prieto M, Castanho M, Coutinho A, Veiga AS (**2019**). The mechanism of action of pepR, a viral-derived peptide, against *Staphylococcus aureus* biofilms. **J Antimicrob Chemother** 74(9): 2617-2625. doi: 10.1093/jac/dkz223.
544. De Man JC, Rogosa, D., & Sharpe, M. E. (**1960**). A medium for the cultivation of lactobacilli. **Journal of applied Bacteriology** 23(1): 5. doi: 10.1111/j.1365-2672.1960.tb00188.x.
545. Roy D (**2001**). Media for the isolation and enumeration of bifidobacteria in dairy products. **International journal of food microbiology** 69(3): 167-182. doi: 10.1016/S0168-1605(01)00496-2.
546. Pramanick R, Mayadeo N, Warke H, Begum S, Aich P, Aranha C (**2019**). Vaginal microbiota of asymptomatic bacterial vaginosis and vulvovaginal candidiasis: Are they different from normal microbiota? **Microb Pathog** 134(103599. doi: 10.1016/j.micpath.2019.103599.
547. Ma B, France MT, Crabtree J, Holm JB, Humphrys MS, Brotman RM, Ravel J (**2020**). A comprehensive non-redundant gene catalog reveals extensive within-community intraspecies diversity in the human vagina. **Nat Commun** 11(1): 940. doi: 10.1038/s41467-020-14677-3.
548. Chee WJY, Chew SY, Than LTL (**2020**). Vaginal microbiota and the potential of *Lactobacillus* derivatives in maintaining vaginal health. **Microb Cell Fact** 19(1): 203. doi: 10.1186/s12934-020-01464-4.
549. Hood S, Zoitola E (**1988**). Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. **Journal of Food Science** 53(5): 1514-1516. doi: 10.1111/j.1365-2621.1988.tb09312.x.
550. Zimmerman T, Gyawali R, Ibrahim S (**2017**). Autolyse the cell in order to save it? Inducing, then blocking, autolysis as a strategy for delaying cell death in the probiotic *Lactobacillus reuteri*. **Biotechnol Lett** 39(10): 1547-1551. doi: 10.1007/s10529-017-2380-8.
551. Kang O, Vézinz L-P, Laberge S, Simard R (**1998**). Some factors influencing the autolysis of *Lactobacillus bulgaricus* and *Lactobacillus casei*. **Journal of Dairy Science** 81(3): 639-646. doi: 10.3168/jds.S0022-0302(98)75618-8.
552. Masuda T, Hidaka A, Kondo N, Ura T, Itoh T (**2005**). Intracellular enzyme activities and autolytic properties of *Lactobacillus acidophilus* and *Lactobacillus gasseri*. **Food science and technology research** 11(3): 328-331. doi: 10.3136/fstr.11.328.
553. Yokoi KJ, Kawasaki K, Taketo A, Kodaira K (**2004**). Characterization of lytic enzyme activities of *Lactobacillus gasseri* with special reference to autolysis. **Int J Food Microbiol** 96(3): 273-279. doi: 10.1016/j.ijfoodmicro.2004.03.021.
554. Ismail EA, Neve H, Geis A, Heller KJ (**2009**). Characterization of temperate *Lactobacillus gasseri* phage LgaI and its impact as prophage on autolysis of its lysogenic host strains. **Curr Microbiol** 58(6): 648-653. doi: 10.1007/s00284-009-9384-0.
555. Goncalves B, Fernandes L, Henriques M, Silva S (**2020**). Environmental pH modulates biofilm formation and matrix composition in *Candida albicans* and *Candida glabrata*. **Biofouling** 36(5): 621-630. doi: 10.1080/08927014.2020.1793963.
556. Ramage G, Walle KV, Wickes BL, Lopez-Ribot JL (**2001**). Characteristics of biofilm formation by *Candida albicans*. **Revista iberoamericana de micología** 18(4): 163-170. doi: PMID: 15496122.
557. Baillie GS, Douglas LJ (**1999**). Role of dimorphism in the development of *Candida albicans* biofilms. **J Med Microbiol** 48(7): 671-679. doi: 10.1099/00222615-48-7-671.
558. Kerstens M, Boulet G, Tritsmans C, Horemans T, Hellings M, Delputte P, Maes L, Cos P (**2014**). Flow cytometric enumeration of bacteria using TO-PRO(R)-3 iodide as a single-stain viability dye. **J Lab Autom** 19(6): 555-561. doi: 10.1177/2211068214546745.
559. Tavecchio M, Simone M, Bernasconi S, Tognon G, Mazzini G, Erba E (**2008**). Multi-parametric flow cytometric cell cycle analysis using TO-PRO-3 iodide (TP3): detailed protocols. **Acta Histochem** 110(3): 232-244. doi: 10.1016/j.acthis.2007.10.007.
560. George J, Halami PM (**2019**). Presence of extracellular DNA & protein in biofilm formation by gentamicin-resistant *Lactobacillus plantarum*. **Indian J Med Res** 149(2): 257-262. doi: 10.4103/ijmr.IJMR_2022_17.
561. Muscariello L, Marino C, Capri U, Vastano V, Marasco R, Sacco M (**2013**). CcpA and three newly identified proteins are involved in biofilm development in *Lactobacillus plantarum*. **J Basic Microbiol** 53(1): 62-71. doi: 10.1002/jobm.201100456.
562. Fernandez Ramirez MD, Smid EJ, Abee T, Nierop Groot MN (**2015**). Characterisation of biofilms formed by *Lactobacillus plantarum* WCFS1 and food spoilage isolates. **Int J Food Microbiol** 207(23-29. doi: 10.1016/j.ijfoodmicro.2015.04.030.

563. Martins M, Uppuluri P, Thomas DP, Cleary IA, Henriques M, Lopez-Ribot JL, Oliveira R (**2010**). Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. **Mycopathologia** 169(5): 323-331. doi: 10.1007/s11046-009-9264-y.
564. Ge J, Kang J, Ping W (**2019**). Effect of Acetic Acid on Bacteriocin Production by Gram-Positive Bacteria. **J Microbiol Biotechnol** 29(9): 1341-1348. doi: 10.4014/jmb.1905.05060.
565. Meng F, Zhao H, Nie T, Lu F, Zhang C, Lu Y, Lu Z (**2021**). Acetate Activates *Lactobacillus* Bacteriocin Synthesis by Controlling Quorum Sensing. **Appl Environ Microbiol** 87(13): e0072021. doi: 10.1128/AEM.00720-21.
566. James KM, MacDonald KW, Chanyi RM, Cadieux PA, Burton JP (**2016**). Inhibition of *Candida albicans* biofilm formation and modulation of gene expression by probiotic cells and supernatant. **J Med Microbiol** 65(4): 328-336. doi: 10.1099/jmm.0.000226.
567. Danhof HA, Vylkova S, Vesely EM, Ford AE, Gonzalez-Garay M, Lorenz MC (**2016**). Robust Extracellular pH Modulation by *Candida albicans* during Growth in Carboxylic Acids. **mBio** 7(6). doi: 10.1128/mBio.01646-16.
568. Vylkova S, Carman AJ, Danhof HA, Collette JR, Zhou H, Lorenz MC (**2011**). The fungal pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising extracellular pH. **mBio** 2(3): e00055-00011. doi: 10.1128/mBio.00055-11.
569. Vylkova S, Lorenz MC (**2014**). Modulation of phagosomal pH by *Candida albicans* promotes hyphal morphogenesis and requires Stp2p, a regulator of amino acid transport. **PLoS Pathog** 10(3): e1003995. doi: 10.1371/journal.ppat.1003995.
570. Moosa MY, Sobel JD, Elhalis H, Du W, Akins RA (**2004**). Fungicidal activity of fluconazole against *Candida albicans* in a synthetic vagina-simulative medium. **Antimicrob Agents Chemother** 48(1): 161-167. doi: 10.1128/AAC.48.1.161-167.2004.
571. Kasper L, Miramon P, Jablonowski N, Wisgott S, Wilson D, Brunke S, Hube B (**2015**). Antifungal activity of clotrimazole against *Candida albicans* depends on carbon sources, growth phase and morphology. **J Med Microbiol** 64(7): 714-723. doi: 10.1099/jmm.0.000082.
572. Vitali B, Cruciani F, Picone G, Parolin C, Donders G, Laghi L (**2015**). Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. **Eur J Clin Microbiol Infect Dis** 34(12): 2367-2376. doi: 10.1007/s10096-015-2490-y.
573. Stiles J, Penkar S, Plockova M, Chumchalova J, Bullerman LB (**2002**). Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. **J Food Prot** 65(7): 1188-1191. doi: 10.4315/0362-028x-65.7.1188.
574. Khoury ZH, Vila T, Puthran TR, Sultan AS, Montelongo-Jauregui D, Melo MAS, Jabra-Rizk MA (**2020**). The Role of *Candida albicans* Secreted Polysaccharides in Augmenting *Streptococcus mutans* Adherence and Mixed Biofilm Formation: *In vitro* and *in vivo* Studies. **Front Microbiol** 11(307. doi: 10.3389/fmicb.2020.00307.
575. Kong EF, Tsui C, Kucharikova S, Andes D, Van Dijck P, Jabra-Rizk MA (**2016**). Commensal Protection of *Staphylococcus aureus* against Antimicrobials by *Candida albicans* Biofilm Matrix. **mBio** 7(5). doi: 10.1128/mBio.01365-16.
576. Bandara HM, Yau JY, Watt RM, Jin LJ, Samaranayake LP (**2010**). *Pseudomonas aeruginosa* inhibits *in vitro Candida* biofilm development. **BMC Microbiol** 10(125. doi: 10.1186/1471-2180-10-125.
577. Yamasaki-Yashiki S, Sawada H, Kino-Oka M, Katakura Y (**2017**). Analysis of gene expression profiles of *Lactobacillus paracasei* induced by direct contact with *Saccharomyces cerevisiae* through recognition of yeast mannan. **Biosci Microbiota Food Health** 36(1): 17-25. doi: 10.12938/bmfh.BMFH-2016-015.
578. Kucharikova S, Tournu H, Lagrou K, Van Dijck P, Bujdakova H (**2011**). Detailed comparison of *Candida albicans* and *Candida glabrata* biofilms under different conditions and their susceptibility to caspofungin and anidulafungin. **J Med Microbiol** 60(Pt 9): 1261-1269. doi: 10.1099/jmm.0.032037-0.
579. Pierce CG, Vila T, Romo JA, Montelongo-Jauregui D, Wall G, Ramasubramanian A, Lopez-Ribot JL (**2017**). The *Candida albicans* Biofilm Matrix: Composition, Structure and Function. **J Fungi (Basel)** 3(1). doi: 10.3390/jof3010014.
580. Salazar SB, Pinheiro MJF, Sotti-Novais D, Soares AR, Lopes MM, Ferreira T, Rodrigues V, Fernandes F, Mira NP (**2022**). Disclosing azole resistance mechanisms in resistant *Candida glabrata* strains encoding wild-type or gain-of-function CgPDR1 alleles through comparative genomics and transcriptomics. **G3 (Bethesda)** 12(7). doi: 10.1093/g3journal/jkac110.
581. Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, Rodloff A, Fu W, Ling TA, Global Antifungal Surveillance G (**2010**). Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* Species to

fluconazole and voriconazole as determined by CLSI standardized disk diffusion. **J Clin Microbiol** 48(4): 1366-1377. doi: 10.1128/JCM.02117-09.
582. Nicolo S, Tanturli M, Mattiuz G, Antonelli A, Baccani I, Bonaiuto C, Baldi S, Nannini G, Menicatti M, Bartolucci G, Rossolini GM, Amedei A, Torcia MG (**2021**). Vaginal Lactobacilli and Vaginal Dysbiosis-Associated Bacteria Differently Affect Cervical Epithelial and Immune Homeostasis and Anti-Viral Defenses. **Int J Mol Sci** 22(12). doi: 10.3390/ijms22126487.
583. Nilsson L, Nielsen MK, Ng Y, Gram L (**2002**). Role of acetate in production of an autoinducible class IIa bacteriocin in *Carnobacterium piscicola* A9b. **Appl Environ Microbiol** 68(5): 2251-2260. doi: 10.1128/AEM.68.5.2251-2260.2002.
584. Kelly W, Asmundson R, Huang C (**1996**). Characterization of plantaricin KW30, a bacteriocin produced by *Lactobacillus plantarum*. **Journal of Applied Bacteriology** 81(6): 657-662. doi: 10.1111/j.1365-2672.1996.tb03561.x.
585. Meng F, Lu F, Du H, Nie T, Zhu X, Connerton IF, Zhao H, Bie X, Zhang C, Lu Z, Lu Y (**2021**). Acetate and auto-inducing peptide are independent triggers of quorum sensing in *Lactobacillus plantarum*. **Mol Microbiol** 116(1): 298-310. doi: 10.1111/mmi.14709.
586. de Jong A, van Heel AJ, Kok J, Kuipers OP (**2010**). BAGEL2: mining for bacteriocins in genomic data. **Nucleic Acids Res** 38(Web Server issue): W647-651. doi: 10.1093/nar/gkq365.
587. Maldonado-Barragán A, West SA (**2020**). The cost and benefit of quorum sensing-controlled bacteriocin production in *Lactobacillus plantarum*. **Journal of evolutionary biology** 33(1): 101-111. doi: 10.1111/jeb.13551.
588. Sobel JD (**2007**). Vulvovaginal candidosis. **The Lancet** 369(9577): 1961-1971. doi: 10.1016/S0140-6736(07)60917-9.
589. Foxman B, Barlow R, D'arcy H, Gillespie B, Sobel JD (**2000**). *Candida* vaginitis: self-reported incidence and associated costs. **Sexually transmitted diseases**: 230-235. doi: 10.1097/00007435-200004000-00009.
590. Sobel JD (**2016**). Recurrent vulvovaginal candidiasis. **Am J Obstet Gynecol** 214(1): 15-21. doi: 10.1016/j.ajog.2015.06.067.
591. Sobel JD (**2002**). Pathogenesis of Recurrent Vulvovaginal Candidiasis. **Curr Infect Dis Rep** 4(6): 514-519. doi: 10.1007/s11908-002-0038-7.
592. Gong X, Luan T, Wu X, Li G, Qiu H, Kang Y, Qin B, Fang Q, Cui W, Qin Y, Li J, Zang B (**2016**). Invasive candidiasis in intensive care units in China: Risk factors and prognoses of *Candida albicans* and non-albicans *Candida* infections. **Am J Infect Control** 44(5): e59-63. doi: 10.1016/j.ajic.2015.11.028.
593. Papon N, Courdavault V, Clastre M, Bennett RJ (**2013**). Emerging and emerged pathogenic Candida species: beyond the Candida albicans paradigm. **PLoS Pathog** 9(9): e1003550. doi: 10.1371/journal.ppat.1003550.
594. Sanguinetti M, Posteraro B, Lass-Florl C (**2015**). Antifungal drug resistance among Candida species: mechanisms and clinical impact. **Mycoses** 58 Suppl 2(2-13. doi: 10.1111/myc.12330.
595. Vilela SF, Barbosa JO, Rossoni RD, Santos JD, Prata MC, Anbinder AL, Jorge AO, Junqueira JC (**2015**). *Lactobacillus acidophilus* ATCC 4356 inhibits biofilm formation by *C. albicans* and attenuates the experimental candidiasis in *Galleria mellonella*. **Virulence** 6(1): 29-39. doi: 10.4161/21505594.2014.981486.
596. Pfaller MA, Andes D, Diekema DJ, Espinel-Ingroff A, Sheehan D, Testing CSfAS (**2010**). Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: time for harmonization of CLSI and EUCAST broth microdilution methods. **Drug Resist Updat** 13(6): 180-195. doi: 10.1016/j.drup.2010.09.002.
597. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, Diekema DJ (**2011**). Wild-type MIC distributions and epidemiological cutoff values for posaconazole and voriconazole and *Candida* spp. as determined by 24-hour CLSI broth microdilution. **J Clin Microbiol** 49(2): 630-637. doi: 10.1128/JCM.02161-10.
598. Ang XY, Chung FY, Lee BK, Azhar SNA, Sany S, Roslan NS, Ahmad N, Yusof SM, Abdullah N, Nik Ab Rahman NN, Abdul Wahid N, Deris ZZ, Oon CE, Wan Adnan WF, Liong MT (**2022**). Lactobacilli reduce recurrences of vaginal candidiasis in pregnant women: a randomized, double-blind, placebo-controlled study. **J Appl Microbiol** 132(4): 3168-3180. doi: 10.1111/jam.15158.
599. Falagas ME, Betsi GI, Athanasiou S (**2006**). Probiotics for prevention of recurrent vulvovaginal candidiasis: a review. **J Antimicrob Chemother** 58(2): 266-272. doi: 10.1093/jac/dkl246.
600. De Gregorio PR, Silva JA, Marchesi A, Nader-Macias MEF (**2019**). Anti-*Candida* activity of beneficial vaginal lactobacilli in *in vitro* assays and in a murine experimental model. **FEMS Yeast Res** 19(2). doi: 10.1093/femsyr/foz008.

601. Chen X, Lu Y, Chen T, Li R (**2021**). The Female Vaginal Microbiome in Health and Bacterial Vaginosis. **Front Cell Infect Microbiol** 11(631972. doi: 10.3389/fcimb.2021.631972.
602. Osset J, Garcia E, Bartolome RM, Andreu A (**2001**). Role of *Lactobacillus* as protector against vaginal candidiasis. **Med Clin (Barc)** 117(8): 285-288. doi: 10.1016/s0025-7753(01)72089-1.
603. Gudina EJ, Teixeira JA, Rodrigues LR (**2010**). Isolation and functional characterization of a biosurfactant produced by *Lactobacillus paracasei*. **Colloids Surf B Biointerfaces** 76(1): 298-304. doi: 10.1016/j.colsurfb.2009.11.008.
604. Gudina EJ, Rocha V, Teixeira JA, Rodrigues LR (**2010**). Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp. paracasei A20. **Lett Appl Microbiol** 50(4): 419-424. doi: 10.1111/j.1472-765X.2010.02818.x.
605. Pedro NA, Fontebasso G, Pinto SN, Alves M, Mira NP (**2023**). Acetate modulates the inhibitory effect of *Lactobacillus gasseri* against the pathogenic yeasts *Candida albicans* and *Candida glabrata*. **Microb Cell** 10(4): 88-102. doi: 10.15698/mic2023.04.795.
606. Black B, Lee C, Horianopoulos LC, Jung WH, Kronstad JW (**2021**). Respiring to infect: Emerging links between mitochondria, the electron transport chain, and fungal pathogenesis. **PLoS Pathog** 17(7): e1009661. doi: 10.1371/journal.ppat.1009661.
607. Su C, Lu Y, Liu H (**2013**). Reduced TOR signaling sustains hyphal development in *Candida albicans* by lowering Hog1 basal activity. **Mol Biol Cell** 24(3): 385-397. doi: 10.1091/mbc.E12-06-0477.
608. Palanisamy SK, Ramirez MA, Lorenz M, Lee SA (**2010**). *Candida albicans* PEP12 is required for biofilm integrity and *in vivo* virulence. **Eukaryot Cell** 9(2): 266-277. doi: 10.1128/EC.00295-09.
609. Westermann AJ, Forstner KU, Amman F, Barquist L, Chao Y, Schulte LN, Muller L, Reinhardt R, Stadler PF, Vogel J (**2016**). Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. **Nature** 529(7587): 496-501. doi: 10.1038/nature16547.
610. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (**2008**). Mapping and quantifying mammalian transcriptomes by RNA-Seq. **Nat Methods** 5(7): 621-628. doi: 10.1038/nmeth.1226.
611. Anders S, Pyl PT, Huber W (**2015**). HTSeq--a Python framework to work with high-throughput sequencing data. **Bioinformatics** 31(2): 166-169. doi: 10.1093/bioinformatics/btu638.
612. McClure R, Balasubramanian D, Sun Y, Bobrovskyy M, Sumby P, Genco CA, Vanderpool CK, Tjaden B (**2013**). Computational analysis of bacterial RNA-Seq data. **Nucleic Acids Res** 41(14): e140. doi: 10.1093/nar/gkt444.
613. Leng N, Dawson JA, Thomson JA, Ruotti V, Rissman AI, Smits BM, Haag JD, Gould MN, Stewart RM, Kendziorski C (**2013**). EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. **Bioinformatics** 29(8): 1035-1043. doi: 10.1093/bioinformatics/btt087.
614. Llorens-Rico V, Serrano L, Lluch-Senar M (**2014**). Assessing the hodgepodge of non-mapped reads in bacterial transcriptomes: real or artifactual RNA chimeras? **BMC Genomics** 15(1): 633. doi: 10.1186/1471-2164-15-633.
615. Siegel TN, Hon CC, Zhang Q, Lopez-Rubio JJ, Scheidig-Benatar C, Martins RM, Sismeiro O, Coppee JY, Scherf A (**2014**). Strand-specific RNA-Seq reveals widespread and developmentally regulated transcription of natural antisense transcripts in *Plasmodium falciparum*. **BMC Genomics** 15(1): 150. doi: 10.1186/1471-2164-15-150.
616. Hovhannisyan H, Gabaldon T (**2021**). The long non-coding RNA landscape of *Candida* yeast pathogens. **Nat Commun** 12(1): 7317. doi: 10.1038/s41467-021-27635-4.
617. Wang J, Zhang J, Liu W, Zhang H, Sun Z (**2021**). Metagenomic and metatranscriptomic profiling of *Lactobacillus casei* Zhang in the human gut. **NPJ Biofilms Microbiomes** 7(1): 55. doi: 10.1038/s41522-021-00227-2.
618. Cousin FJ, Lynch DB, Chuat V, Bourin MJB, Casey PG, Dalmasso M, Harris HMB, McCann A, O'Toole PW (**2017**). A long and abundant non-coding RNA in *Lactobacillus salivarius*. **Microb Genom** 3(9): e000126. doi: 10.1099/mgen.0.000126.
619. Timmermans B, De Las Penas A, Castano I, Van Dijck P (**2018**). Adhesins in *Candida glabrata*. **J Fungi (Basel)** 4(2). doi: 10.3390/jof4020060.
620. Whaley SG, Rogers PD (**2016**). Azole Resistance in *Candida glabrata*. **Curr Infect Dis Rep** 18(12): 41. doi: 10.1007/s11908-016-0554-5.
621. Yu X, Mao Y, Li G, Wu X, Xuan Q, Yang S, Chen X, Cao Q, Guo J, Guo J (**2023**). Alpha-Hemolysin from *Staphylococcus aureus* Obstructs Yeast-Hyphae Switching and Diminishes Pathogenicity in *Candida albicans*. **Journal of Microbiology**: 1-11. doi: 10.1007/s12275-022-00006-4.

622. Ruiz A, González A, Muñoz I, Serrano R, Abrie JA, Strauss E, Ariño J (**2009**). Moonlighting proteins Hal3 and Vhs3 form a heteromeric PPCDC with Ykl088w in yeast CoA biosynthesis. **Nature chemical biology** 5(12): 920-928. doi: 10.1038/nchembio.243.
623. Zhang J, Smith KM, Tackaberry T, Sun X, Carpenter P, Slugoski MD, Robins MJ, Nielsen LP, Nowak I, Baldwin SA, Young JD, Cass CE (**2006**). Characterization of the transport mechanism and permeant binding profile of the uridine permease Fui1p of *Saccharomyces cerevisiae*. **J Biol Chem** 281(38): 28210-28221. doi: 10.1074/jbc.M605129200.
624. Pais P, California R, Galocha M, Viana R, Ola M, Cavalheiro M, Takahashi-Nakaguchi A, Chibana H, Butler G, Teixeira MC (**2020**). *Candida glabrata* Transcription Factor Rpn4 Mediates Fluconazole Resistance through Regulation of Ergosterol Biosynthesis and Plasma Membrane Permeability. **Antimicrob Agents Chemother** 64(9). doi: 10.1128/AAC.00554-20.
625. Ng TS, Mohd Desa MN, Sandai D, Chong PP, Than LT (**2015**). Phylogenetic and Transcripts Profiling of Glucose Sensing Related Genes in *Candida glabrata*. **Jundishapur J Microbiol** 8(11): e25177. doi: 10.5812/jjm.25177.
626. van Zanten GC, Sparding N, Majumder A, Lahtinen SJ, Svensson B, Jacobsen S (**2015**). The Differential Proteome of the Probiotic *Lactobacillus acidophilus* NCFM Grown on the Potential Prebiotic Cellobiose Shows Upregulation of Two beta -Glycoside Hydrolases. **Biomed Res Int** 2015(347216. doi: 10.1155/2015/347216.
627. Benchouaia M, Ripoche H, Sissoko M, Thiebaut A, Merhej J, Delaveau T, Fasseu L, Benaissa S, Lorieux G, Jourdren L, Le Crom S, Lelandais G, Corel E, Devaux F (**2018**). Comparative Transcriptomics Highlights New Features of the Iron Starvation Response in the Human Pathogen *Candida glabrata*. **Front Microbiol** 9(2689. doi: 10.3389/fmicb.2018.02689.
628. Imbert M, Blondeau R (**1998**). On the iron requirement of lactobacilli grown in chemically defined medium. **Curr Microbiol** 37(1): 64-66. doi: 10.1007/s002849900339.
629. Weinberg ED (**1997**). The *Lactobacillus* anomaly: total iron abstinence. **Perspect Biol Med** 40(4): 578-583. doi: 10.1353/pbm.1997.0072.
630. Sedat Ç (**2020**). The effect of iron on the expression of hemolysin/cytolysin and growth of clinical and environmental strains of *Vibrio vulnificus*. **Etlik Veteriner Mikrobiyoloji Dergisi** 31(2): 121-126. doi: 10.35864/evmd.788440.
631. Teng T, Xi B, Chen K, Pan L, Xie J, Xu P (**2018**). Comparative transcriptomic and proteomic analyses reveal upregulated expression of virulence and iron transport factors of *Aeromonas hydrophila* under iron limitation. **BMC Microbiol** 18(1): 52. doi: 10.1186/s12866-018-1178-8.
632. Torres VJ, Attia AS, Mason WJ, Hood MI, Corbin BD, Beasley FC, Anderson KL, Stauff DL, McDonald WH, Zimmerman LJ, Friedman DB, Heinrichs DE, Dunman PM, Skaar EP (**2010**). *Staphylococcus aureus* fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. **Infect Immun** 78(4): 1618-1628. doi: 10.1128/IAI.01423-09.
633. Hertzberger R, Arents J, Dekker HL, Pridmore RD, Gysler C, Kleerebezem M, de Mattos MJ (**2014**). H_2O_2 production in species of the *Lactobacillus acidophilus* group: a central role for a novel NADH-dependent flavin reductase. **Appl Environ Microbiol** 80(7): 2229-2239. doi: 10.1128/AEM.04272-13.
634. Pridmore RD, Pittet AC, Praplan F, Cavadini C (**2008**). Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. **FEMS Microbiol Lett** 283(2): 210-215. doi: 10.1111/j.1574-6968.2008.01176.x.
635. Beyer R, Jandric Z, Zutz C, Gregori C, Willinger B, Jacobsen ID, Kovarik P, Strauss J, Schuller C (**2018**). Competition of *Candida glabrata* against Lactobacillus is Hog1 dependent. **Cell Microbiol** 20(12): e12943. doi: 10.1111/cmi.12943.
636. Tanaka K, Tatebayashi K, Nishimura A, Yamamoto K, Yang HY, Saito H (**2014**). Yeast osmosensors Hkr1 and Msb2 activate the Hog1 MAPK cascade by different mechanisms. **Sci Signal** 7(314): ra21. doi: 10.1126/scisignal.2004780.
637. de Nadal E, Posas F (**2022**). The HOG pathway and the regulation of osmoadaptive responses in yeast. **FEMS Yeast Res** 22(1). doi: 10.1093/femsyr/foac013.
638. Calafi C, Lopez-Malo M, Velazquez D, Zhang C, Fernandez-Fernandez J, Rodriguez-Galan O, de la Cruz J, Arino J, Casamayor A (**2020**). Overexpression of budding yeast protein phosphatase Ppz1 impairs translation. **Biochim Biophys Acta Mol Cell Res** 1867(8): 118727. doi: 10.1016/j.bbamcr.2020.118727.
639. Siles SA, Srinivasan A, Pierce CG, Lopez-Ribot JL, Ramasubramanian AK (**2013**). High-throughput screening of a collection of known pharmacologically active small compounds for identification of *Candida albicans* biofilm inhibitors. **Antimicrob Agents Chemother** 57(8): 3681-3687. doi: 10.1128/AAC.00680-13.

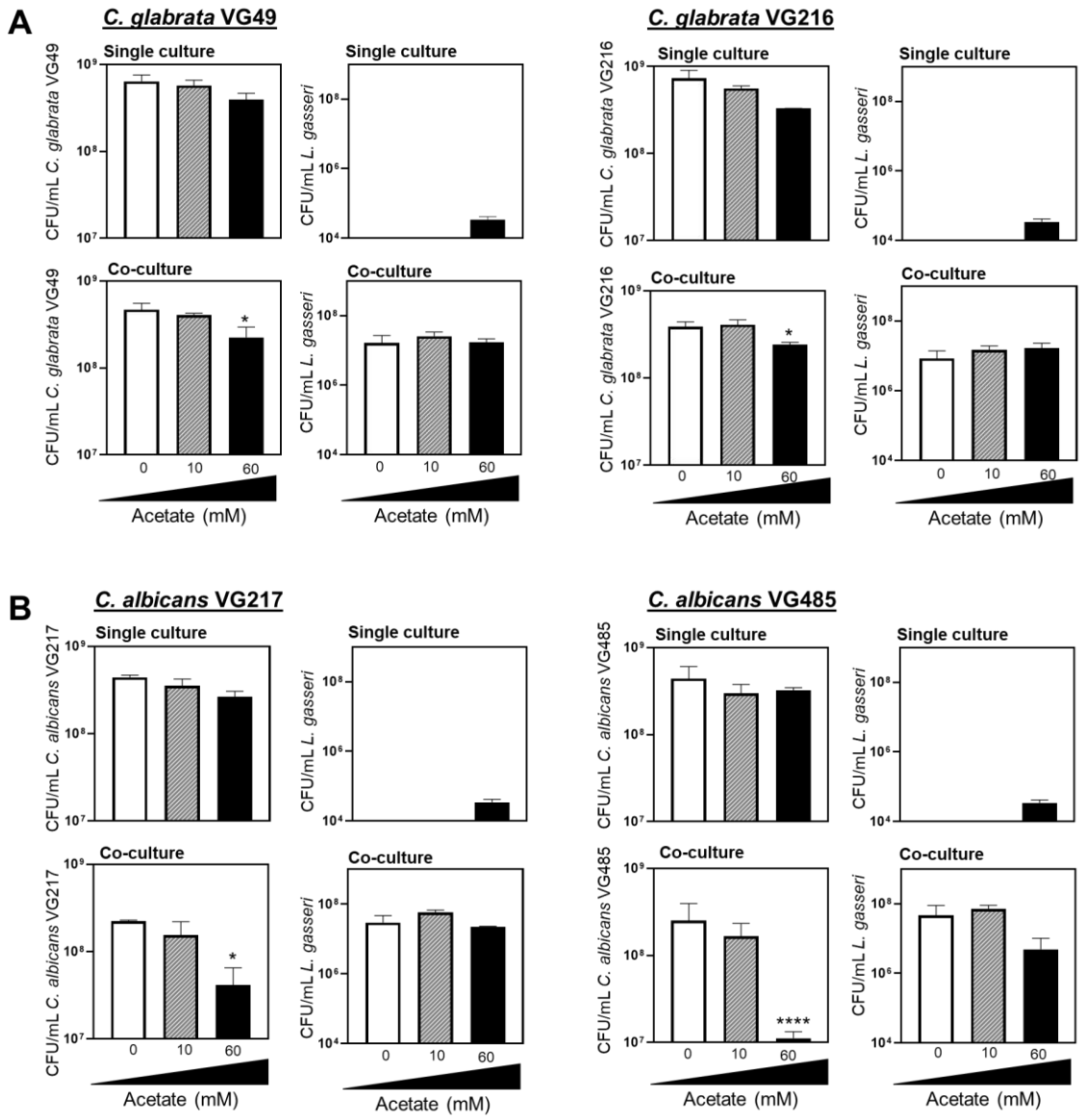
640. Vazquez-Ibarra A, Subirana L, Ongay-Larios L, Kawasaki L, Rojas-Ortega E, Rodriguez-Gonzalez M, de Nadal E, Posas F, Coria R (**2018**). Activation of the Hog1 MAPK by the Ssk2/Ssk22 MAP3Ks, in the absence of the osmosensors, is not sufficient to trigger osmostress adaptation in *Saccharomyces cerevisiae*. **FEBS J** 285(6): 1079-1096. doi: 10.1111/febs.14385.
641. Gregori C, Schuller C, Roetzer A, Schwarzmuller T, Ammerer G, Kuchler K (**2007**). The high-osmolarity glycerol response pathway in the human fungal pathogen *Candida glabrata* strain ATCC 2001 lacks a signaling branch that operates in baker's yeast. **Eukaryot Cell** 6(9): 1635-1645. doi: 10.1128/EC.00106-07.
642. Jandric Z, Gregori C, Klopf E, Radolf M, Schuller C (**2013**). Sorbic acid stress activates the *Candida glabrata* high osmolarity glycerol MAP kinase pathway. **Front Microbiol** 4(350. doi: 10.3389/fmicb.2013.00350.
643. Srivastava VK, Suneetha KJ, Kaur R (**2015**). The mitogen-activated protein kinase CgHog1 is required for iron homeostasis, adherence and virulence in *Candida glabrata*. **FEBS J** 282(11): 2142-2166. doi: 10.1111/febs.13264.
644. Schwarzmuller T, Ma B, Hiller E, Istel F, Tscherner M, Brunke S, Ames L, Firon A, Green B, Cabral V, Marcet-Houben M, Jacobsen ID, Quintin J, Seider K, Frohner I, Glaser W, Jungwirth H, Bachellier-Bassi S, Chauvel M, Zeidler U, Ferrandon D, Gabaldon T, Hube B, d'Enfert C, Rupp S, Cormack B, Haynes K, Kuchler K (**2014**). Systematic phenotyping of a large-scale *Candida glabrata* deletion collection reveals novel antifungal tolerance genes. **PLoS Pathog** 10(6): e1004211. doi: 10.1371/journal.ppat.1004211.
645. Robinson DG, Chen W, Storey JD, Gresham D (**2014**). Design and analysis of Bar-seq experiments. **G3 (Bethesda)** 4(1): 11-18. doi: 10.1534/g3.113.008565.
646. Pierce SE, Davis RW, Nislow C, Giaever G (**2007**). Genome-wide analysis of barcoded *Saccharomyces cerevisiae* gene-deletion mutants in pooled cultures. **Nat Protoc** 2(11): 2958-2974. doi: 10.1038/nprot.2007.427.
647. Smith AM, Heisler LE, St Onge RP, Farias-Hesson E, Wallace IM, Bodeau J, Harris AN, Perry KM, Giaever G, Pourmand N, Nislow C (**2010**). Highly-multiplexed barcode sequencing: an efficient method for parallel analysis of pooled samples. **Nucleic Acids Res** 38(13): e142. doi: 10.1093/nar/gkq368.
648. Crowley LC, Scott AP, Marfell BJ, Boughaba JA, Chojnowski G, Waterhouse NJ (**2016**). Measuring cell death by propidium iodide uptake and flow cytometry. **Cold Spring Harbor Protocols** 2016(7): pdb. prot087163. doi: 10.1101/pdb.prot087163.
649. Saijo T, Miyazaki T, Izumikawa K, Mihara T, Takazono T, Kosai K, Imamura Y, Seki M, Kakeya H, Yamamoto Y, Yanagihara K, Kohno S (**2010**). Skn7p is involved in oxidative stress response and virulence of *Candida glabrata*. **Mycopathologia** 169(2): 81-90. doi: 10.1007/s11046-009-9233-5.
650. Wu J, Chen X, Cai L, Tang L, Liu L (**2015**). Transcription factors Asg1p and Hal9p regulate pH homeostasis in *Candida glabrata*. **Front Microbiol** 6(843. doi: 10.3389/fmicb.2015.00843.
651. Muir A (**2015**). Systematic identification of proteins regulated by the TOR Complex 2-dependent kinase Ypk1 in *Saccharomyces cerevisiae*. University of California, Berkeley.
652. de Nadal E, Casadome L, Posas F (**2003**). Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. **Mol Cell Biol** 23(1): 229-237. doi: 10.1128/MCB.23.1.229-237.2003.
653. Cavalheiro M, Costa C, Silva-Dias A, Miranda IM, Wang C, Pais P, Pinto SN, Mil-Homens D, Sato-Okamoto M, Takahashi-Nakaguchi A, Silva RM, Mira NP, Fialho AM, Chibana H, Rodrigues AG, Butler G, Teixeira MC (**2019**). A Transcriptomics Approach To Unveiling the Mechanisms of In Vitro Evolution towards Fluconazole Resistance of a *Candida glabrata* Clinical Isolate. **Antimicrob Agents Chemother** 63(1). doi: 10.1128/AAC.00995-18.
654. Ferraz L, Vorauer-Uhl K, Sauer M, Sousa MJ, Branduardi P (**2023**). Impact of ergosterol content on acetic and lactic acids toxicity to *Saccharomyces cerevisiae*. **Yeast** 40(3-4): 152-165. doi: 10.1002/yea.3828.
655. Fagerlind MG, Webb JS, Barraud N, McDougald D, Jansson A, Nilsson P, Harlen M, Kjelleberg S, Rice SA (**2012**). Dynamic modelling of cell death during biofilm development. **J Theor Biol** 295(23-36. doi: 10.1016/j.jtbi.2011.10.007.
656. Wu C, Zhang J, Zhu G, Yao R, Chen X, Liu L (**2019**). CgHog1-Mediated CgRds2 Phosphorylation Alters Glycerophospholipid Composition To Coordinate Osmotic Stress in *Candida glabrata*. **Appl Environ Microbiol** 85(6). doi: 10.1128/AEM.02822-18.
657. Roelants FM, Torrance PD, Bezman N, Thorner J (**2002**). Pkh1 and Pkh2 differentially phosphorylate and activate Ypk1 and Ykr2 and define protein kinase modules required for maintenance of cell wall integrity. **Mol Biol Cell** 13(9): 3005-3028. doi: 10.1091/mbc.e02-04-0201.

658. Ceballos Garzon A, Amado D, Robert E, Parra Giraldo CM, Le Pape P (**2020**). Impact of calmodulin inhibition by fluphenazine on susceptibility, biofilm formation and pathogenicity of caspofungin-resistant *Candida glabrata*. **J Antimicrob Chemother** 75(5): 1187-1193. doi: 10.1093/jac/dkz565.
659. Chen YL, Brand A, Morrison EL, Silao FG, Bigol UG, Malbas FF, Jr., Nett JE, Andes DR, Solis NV, Filler SG, Averette A, Heitman J (**2011**). Calcineurin controls drug tolerance, hyphal growth, and virulence in *Candida dubliniensis*. **Eukaryot Cell** 10(6): 803-819. doi: 10.1128/EC.00310-10.
660. Juvvadi PR, Lee SC, Heitman J, Steinbach WJ (**2017**). Calcineurin in fungal virulence and drug resistance: Prospects for harnessing targeted inhibition of calcineurin for an antifungal therapeutic approach. **Virulence** 8(2): 186-197. doi: 10.1080/21505594.2016.1201250.
661. Xu H, Fang T, Omran RP, Whiteway M, Jiang L (**2020**). RNA sequencing reveals an additional Crz1-binding motif in promoters of its target genes in the human fungal pathogen *Candida albicans*. **Cell Commun Signal** 18(1): 1. doi: 10.1186/s12964-019-0473-9.
662. Chen YL, Konieczka JH, Springer DJ, Bowen SE, Zhang J, Silao FG, Bungay AA, Bigol UG, Nicolas MG, Abraham SN, Thompson DA, Regev A, Heitman J (**2012**). Convergent Evolution of Calcineurin Pathway Roles in Thermotolerance and Virulence in *Candida glabrata*. **G3 (Bethesda)** 2(6): 675-691. doi: 10.1534/g3.112.002279.
663. Miyazaki T, Yamauchi S, Inamine T, Nagayoshi Y, Saijo T, Izumikawa K, Seki M, Kakeya H, Yamamoto Y, Yanagihara K, Miyazaki Y, Kohno S (**2010**). Roles of calcineurin and Crz1 in antifungal susceptibility and virulence of *Candida glabrata*. **Antimicrob Agents Chemother** 54(4): 1639-1643. doi: 10.1128/AAC.01364-09.
664. Thewes S (**2014**). Calcineurin-Crz1 signaling in lower eukaryotes. **Eukaryot Cell** 13(6): 694-705. doi: 10.1128/EC.00038-14.
665. Chen Y-L, Kozubowski L, Cardenas ME, Heitman J (**2010**). On the roles of calcineurin in fungal growth and pathogenesis. **Current Fungal Infection Reports** 4(244-255. doi: 10.1007/s12281-010-0027-5.
666. Karababa M, Valentino E, Pardini G, Coste AT, Bille J, Sanglard D (**2006**). CRZ1, a target of the calcineurin pathway in *Candida albicans*. **Mol Microbiol** 59(5): 1429-1451. doi: 10.1111/j.1365-2958.2005.05037.x.
667. Armougom F, Henry M, Vialettes B, Raccah D, Raoult D (**2009**). Monitoring bacterial community of human gut microbiota reveals an increase in *Lactobacillus* in obese patients and Methanogens in anorexic patients. **PLoS One** 4(9): e7125. doi: 10.1371/journal.pone.0007125.
668. Zheng J, Wittouck S, Salvetti E, Franz C, Harris HMB, Mattarelli P, O'Toole PW, Pot B, Vandamme P, Walter J, Watanabe K, Wuyts S, Felis GE, Ganzle MG, Lebeer S (**2020**). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. **Int J Syst Evol Microbiol** 70(4): 2782-2858. doi: 10.1099/ijsem.0.004107.
669. Lepargneur JP (**2016**). *Lactobacillus crispatus* as biomarker of the healthy vaginal tract. **Ann Biol Clin (Paris)** 74(4): 421-427. doi: 10.1684/abc.2016.1169.
670. Nissen L, Chingwaru W, Sgorbati B, Biavati B, Cencic A (**2009**). Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. strains: a functional study in the small intestinal cell model. **Int J Food Microbiol** 135(3): 288-294. doi: 10.1016/j.ijfoodmicro.2009.08.027.
671. Mu Q, Tavella VJ, Luo XM (**2018**). Role of *Lactobacillus reuteri* in Human Health and Diseases. **Front Microbiol** 9(757. doi: 10.3389/fmicb.2018.00757.
672. Savino F, Fornasero S, Ceratto S, De Marco A, Mandras N, Roana J, Tullio V, Amisano G (**2015**). Probiotics and gut health in infants: A preliminary case-control observational study about early treatment with *Lactobacillus reuteri* DSM 17938. **Clin Chim Acta** 451(Pt A): 82-87. doi: 10.1016/j.cca.2015.02.027.
673. Oliva S, Di Nardo G, Ferrari F, Mallardo S, Rossi P, Patrizi G, Cucchiara S, Stronati L (**2012**). Randomised clinical trial: the effectiveness of *Lactobacillus reuteri* ATCC 55730 rectal enema in children with active distal ulcerative colitis. **Aliment Pharmacol Ther** 35(3): 327-334. doi: 10.1111/j.1365-2036.2011.04939.x.
674. Ojetti V, Ianiro G, Tortora A, D'Angelo G, Di Rienzo TA, Bibbo S, Migneco A, Gasbarrini A (**2014**). The effect of *Lactobacillus reuteri* supplementation in adults with chronic functional constipation: a randomized, double-blind, placebo-controlled trial. **J Gastrointestin Liver Dis** 23(4): 387-391. doi: 10.15403/jgld.2014.1121.234.elr.

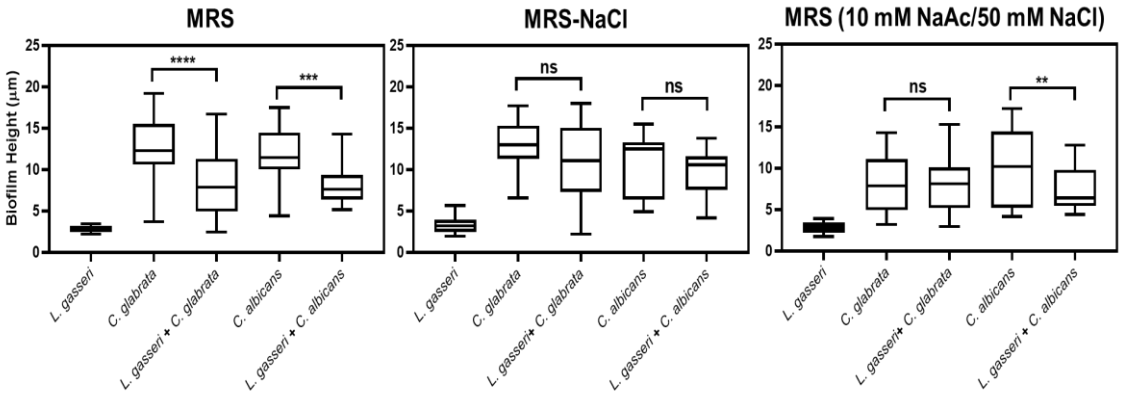
675. Tubelius P, Stan V, Zachrisson A (**2005**). Increasing work-place healthiness with the probiotic *Lactobacillus reuteri*: a randomised, double-blind placebo-controlled study. **Environ Health** 4(25. doi: 10.1186/1476-069X-4-25.
676. Indrio F, Riezzo G, Raimondi F, Bisceglia M, Cavallo L, Francavilla R (**2008**). The effects of probiotics on feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns. **J Pediatr** 152(6): 801-806. doi: 10.1016/j.jpeds.2007.11.005.
677. Spinler JK, Taweechotipatr M, Rognerud CL, Ou CN, Tumwasorn S, Versalovic J (**2008**). Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. **Anaerobe** 14(3): 166-171. doi: 10.1016/j.anaerobe.2008.02.001.
678. Romeo MG, Romeo DM, Trovato L, Oliveri S, Palermo F, Cota F, Betta P (**2011**). Role of probiotics in the prevention of the enteric colonization by *Candida* in preterm newborns: incidence of late-onset sepsis and neurological outcome. **J Perinatol** 31(1): 63-69. doi: 10.1038/jp.2010.57.
679. Bakhshi M, Salari S, Almani PGN, Afshari SAK (**2021**). Evaluation of the antifungal activity of *Lactobacillus reuteri* against *Candida* species. **Gene Reports** 25(101369. doi: 10.1016/j.genrep.2021.101369.
680. Chew SY, Cheah YK, Seow HF, Sandai D, Than LT (**2015**). Probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 exhibit strong antifungal effects against vulvovaginal candidiasis-causing *Candida glabrata* isolates. **J Appl Microbiol** 118(5): 1180-1190. doi: 10.1111/jam.12772.
681. Boahen A, Chew SY, Neela VK, Than LTL (**2023**). *Limosilactobacillus reuteri* 29A Cell-Free Supernatant Antibiofilm and Antagonistic Effects in Murine Model of Vulvovaginal Candidiasis. **Probiotics Antimicrob Proteins**. doi: 10.1007/s12602-023-10050-0.
682. Sims IM, Frese SA, Walter J, Loach D, Wilson M, Appleyard K, Eason J, Livingston M, Baird M, Cook G, Tannock GW (**2011**). Structure and functions of exopolysaccharide produced by gut commensal *Lactobacillus reuteri* 100-23. **ISME J** 5(7): 1115-1124. doi: 10.1038/ismej.2010.201.
683. Arskold E, Svensson M, Grage H, Roos S, Radstrom P, van Niel EW (**2007**). Environmental influences on exopolysaccharide formation in *Lactobacillus reuteri* ATCC 55730. **Int J Food Microbiol** 116(1): 159-167. doi: 10.1016/j.ijfoodmicro.2006.12.010.
684. Widyarman AS, Halim LA, Jesslyn, Irma HA, Richi M, Rizal MI (**2023**). The potential of reuterin derived from Indonesian strain of *Lactobacillus reuteri* against endodontic pathogen biofilms *in vitro* and *ex vivo*. **Saudi Dent J** 35(2): 154-164. doi: 10.1016/j.sdentj.2023.01.002.
685. Maione A, Imparato M, Buonanno A, Carraturo F, Schettino A, Schettino MT, Galdiero M, de Alteriis E, Guida M, Galdiero E (**2023**). Anti-Biofilm Activity of Phenyllactic Acid against Clinical Isolates of Fluconazole-Resistant *Candida albicans*. **J Fungi (Basel)** 9(3). doi: 10.3390/jof9030355.
686. Mil-Homens D, Bernardes N, Fialho AM (**2012**). The antibacterial properties of docosahexaenoic omega-3 fatty acid against the cystic fibrosis multiresistant pathogen *Burkholderia cenocepacia*. **FEMS Microbiol Lett** 328(1): 61-69. doi: 10.1111/j.1574-6968.2011.02476.x.
687. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG (**2011**). *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. **Nat Rev Microbiol** 10(2): 112-122. doi: 10.1038/nrmicro2711.
688. Davis D, Wilson RB, Mitchell AP (**2000**). RIM101-dependent and-independent pathways govern pH responses in *Candida albicans*. **Mol Cell Biol** 20(3): 971-978. doi: 10.1128/MCB.20.3.971-978.2000.
689. Wakade RS, Kramara J, Wellington M, Krysan DJ (**2022**). *Candida albicans* Filamentation Does Not Require the cAMP-PKA Pathway *In Vivo*. **mBio** 13(3): e0085122. doi: 10.1128/mbio.00851-22.
690. Dicksved J, Schreiber O, Willing B, Petersson J, Rang S, Phillipson M, Holm L, Roos S (**2012**). *Lactobacillus reuteri* maintains a functional mucosal barrier during DSS treatment despite mucus layer dysfunction. **PLoS One** 7(9): e46399. doi: 10.1371/journal.pone.0046399.
691. Miniello VL, Brunetti L, Tesse R, Natile M, Armenio L, Francavilla R (**2010**). *Lactobacillus reuteri* modulates cytokines production in exhaled breath condensate of children with atopic dermatitis. **J Pediatr Gastroenterol Nutr** 50(5): 573-576. doi: 10.1097/MPG.0b013e3181bb343f.
692. Kohler GA, Assefa S, Reid G (**2012**). Probiotic interference of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 with the opportunistic fungal pathogen *Candida albicans*. **Infect Dis Obstet Gynecol** 2012(636474. doi: 10.1155/2012/636474.
693. Rossoni RD, Dos Santos Velloso M, Figueiredo LMA, Martins CP, Jorge AOC, Junqueira JC (**2018**). Clinical strains of *Lactobacillus* reduce the filamentation of *Candida albicans* and protect

Galleria mellonella against experimental candidiasis. **Folia Microbiol (Praha)** 63(3): 307-314. doi: 10.1007/s12223-017-0569-9.
694. Rossoni RD, Fuchs BB, de Barros PP, Velloso MD, Jorge AO, Junqueira JC, Mylonakis E (**2017**). *Lactobacillus paracasei* modulates the immune system of *Galleria mellonella* and protects against *Candida albicans* infection. **PLoS One** 12(3): e0173332. doi: 10.1371/journal.pone.0173332.
695. Homayouni A, Bastani P, Ziyadi S, Mohammad-Alizadeh-Charandabi S, Ghalibaf M, Mortazavian AM, Mehrabany EV (**2014**). Effects of probiotics on the recurrence of bacterial vaginosis: a review. **J Low Genit Tract Dis** 18(1): 79-86. doi: 10.1097/LGT.0b013e31829156ec.
696. Dols JA, Molenaar D, van der Helm JJ, Caspers MP, de Kat Angelino-Bart A, Schuren FH, Speksnijder AG, Westerhoff HV, Richardus JH, Boon ME, Reid G, de Vries HJ, Kort R (**2016**). Molecular assessment of bacterial vaginosis by *Lactobacillus* abundance and species diversity. **BMC Infect Dis** 16(180. doi: 10.1186/s12879-016-1513-3.
697. Kotrba P, Inui M, Yukawa H (**2001**). Bacterial phosphotransferase system (PTS) in carbohydrate uptake and control of carbon metabolism. **J Biosci Bioeng** 92(6): 502-517. doi: 10.1263/jbb.92.502.
698. Francl AL, Thongaram T, Miller MJ (**2010**). The PTS transporters of *Lactobacillus gasseri* ATCC 33323. **BMC Microbiol** 10(77. doi: 10.1186/1471-2180-10-77.
699. Briones-Martin-Del-Campo M, Orta-Zavalza E, Juarez-Cepeda J, Gutierrez-Escobedo G, Canas-Villamar I, Castano I, De Las Penas A (**2014**). The oxidative stress response of the opportunistic fungal pathogen *Candida glabrata*. **Rev Iberoam Micol** 31(1): 67-71. doi: 10.1016/j.riam.2013.09.012.
700. Fassler JS, West AH (**2011**). Fungal Skn7 stress responses and their relationship to virulence. **Eukaryot Cell** 10(2): 156-167. doi: 10.1128/EC.00245-10.
701. Dailin DJ, Selvamani S, Michelle K, Jusoh YMM, Chuah LF, Bokhari A, El Enshasy HA, Mubashir M, Show PL (**2022**). Production of high-value added exopolysaccharide by biotherapeutic potential *Lactobacillus reuteri* strain. **Biochemical Engineering Journal** 188(108691. doi: 10.1016/j.bej.2022.108691.
702. Endo A, Futagawa-Endo Y, Kawasaki S, Dicks LM, Niimura Y, Okada S (**2009**). Sodium acetate enhances hydrogen peroxide production in *Weissella cibaria*. **Lett Appl Microbiol** 49(1): 136-141. doi: 10.1111/j.1472-765X.2009.02633.x.

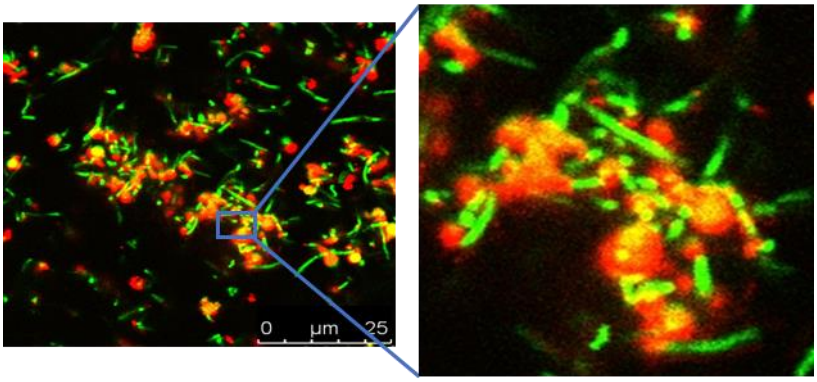
Appendix - Supplementary data



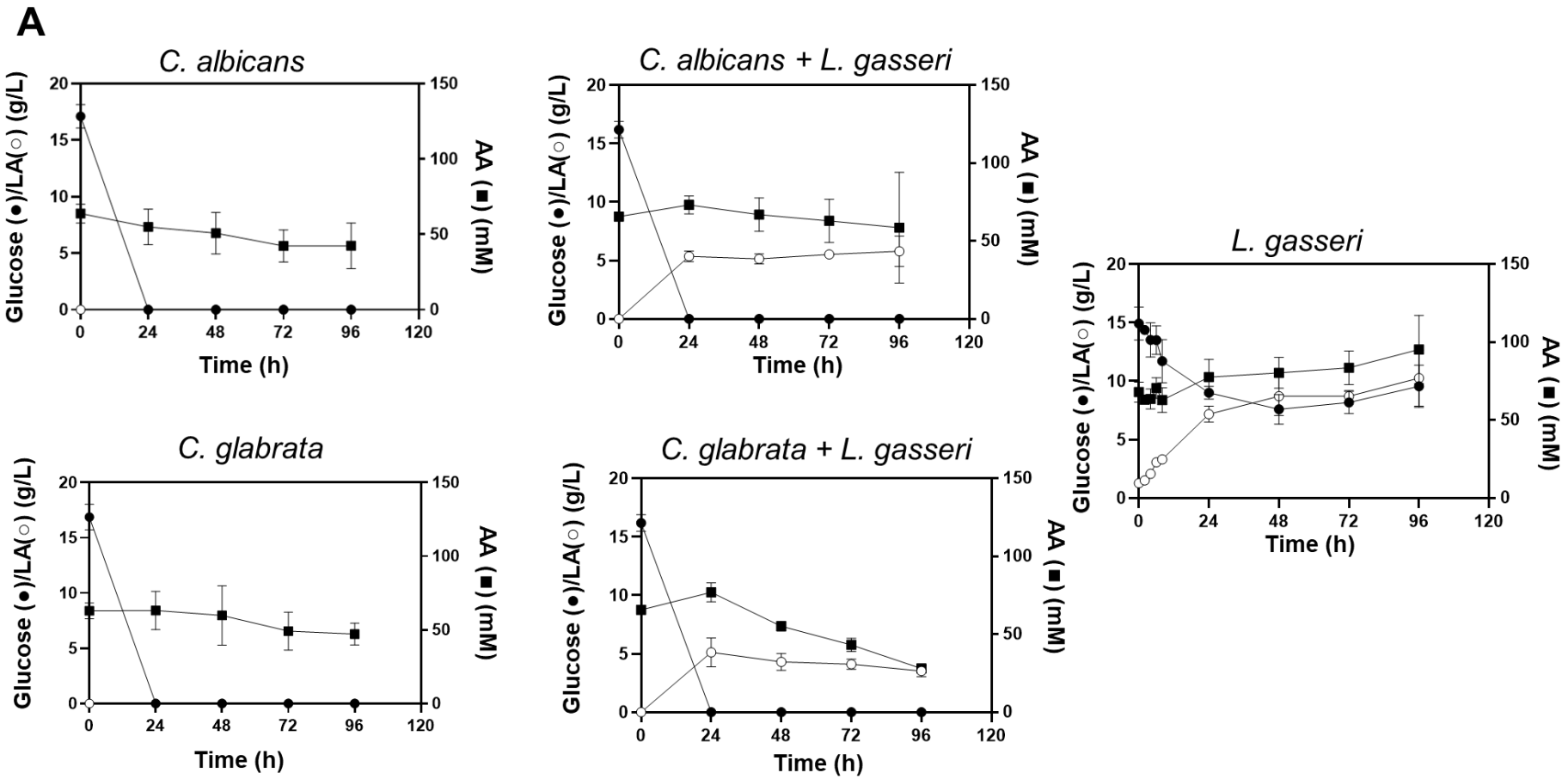
Annex Figure II.1. Viability of vaginal *C. glabrata* **(A)** and *C. albicans* **(B**) vaginal strains after 96h of single or co-cultivation with *L. gasseri* in MRS medium (corresponding to a concentration of acetate of 60 mM) or in this same medium having 60 mM NaCl (in replacement of the sodium acetate) or 10 mM sodium acetate and 50 mM NaCl. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).



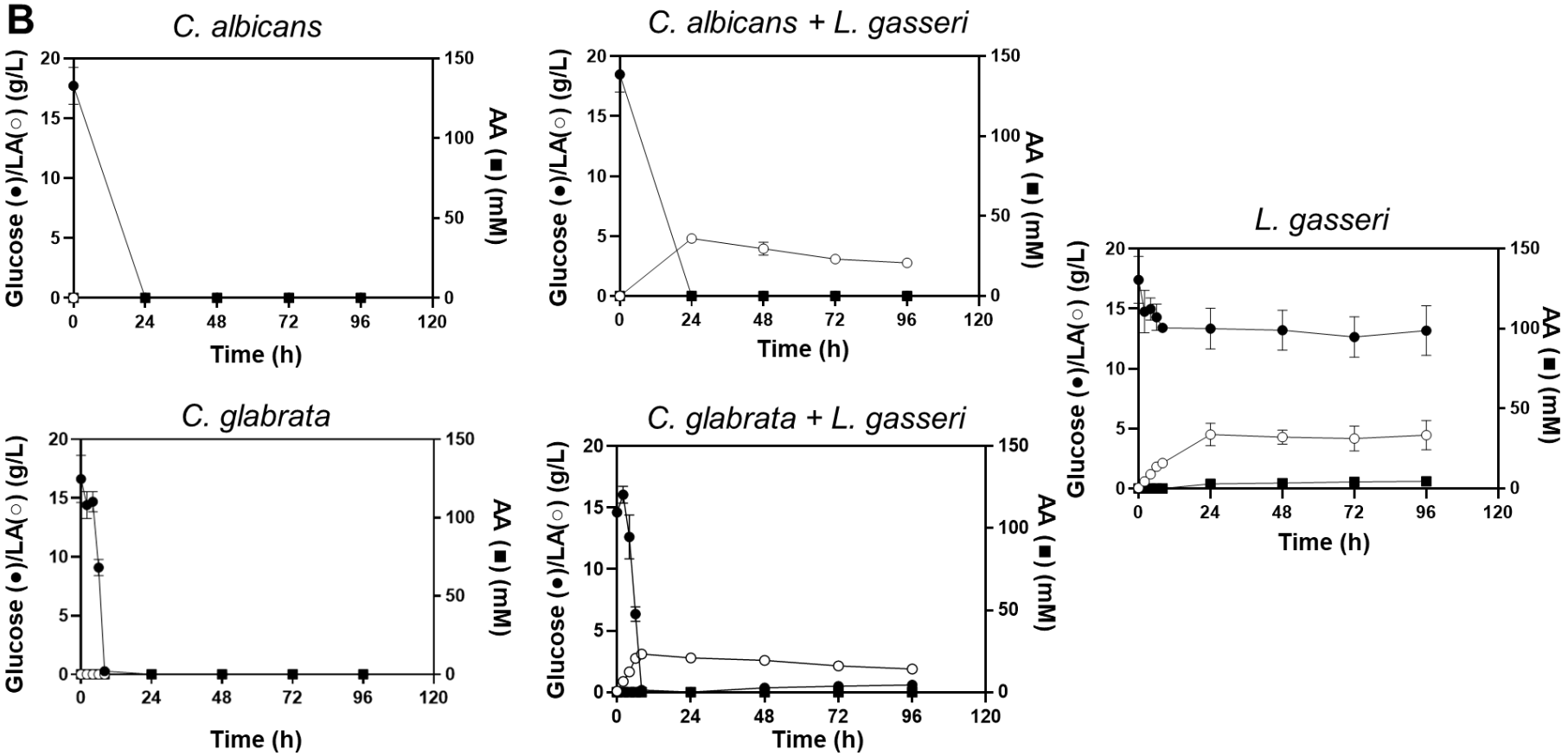
Annex Figure II.2. Height of the biofilms (in µm) formed by *L. gasseri*, *C. albicans* and *C. glabrata* when cultivated, for 24h, alone or in combination in MRS medium containing 60 mM of NaCl (in replacement of sodium acetate) or containing 10 mM sodium acetate (NaAce) and 50 mM NaCl. After cultivation in 8-well microplates, the single and multi-species biofilms were washed, labelled with SYTO9, fixed and imaged by confocal microscopy, as detailed in materials and methods. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).



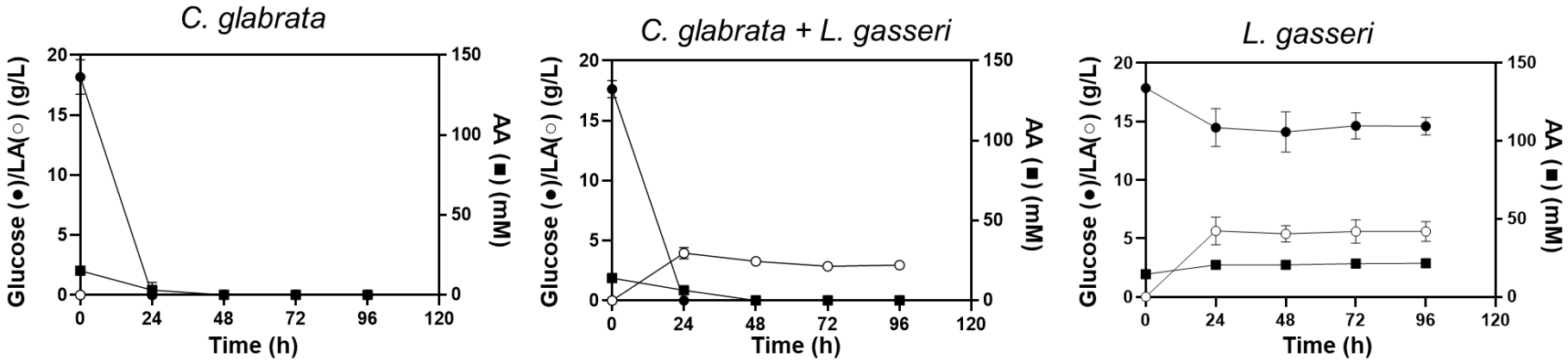
Annex Figure II.3. Detail on the imaging obtained after the overlay of the SYTO9 and TO-PRO-3 Iodide labelling of biofilms formed by *L. gasseri* after 24h of cultivation in MRS medium, as detailed in Figure II.3. Note the marked red accumulation outside of the bacterial cells, in what appears to be the extracellular matrix of the biofilm. These images were obtained after 24h of cultivation at 37ºC and 25 rpm. Scale bar corresponds to 25 µm.



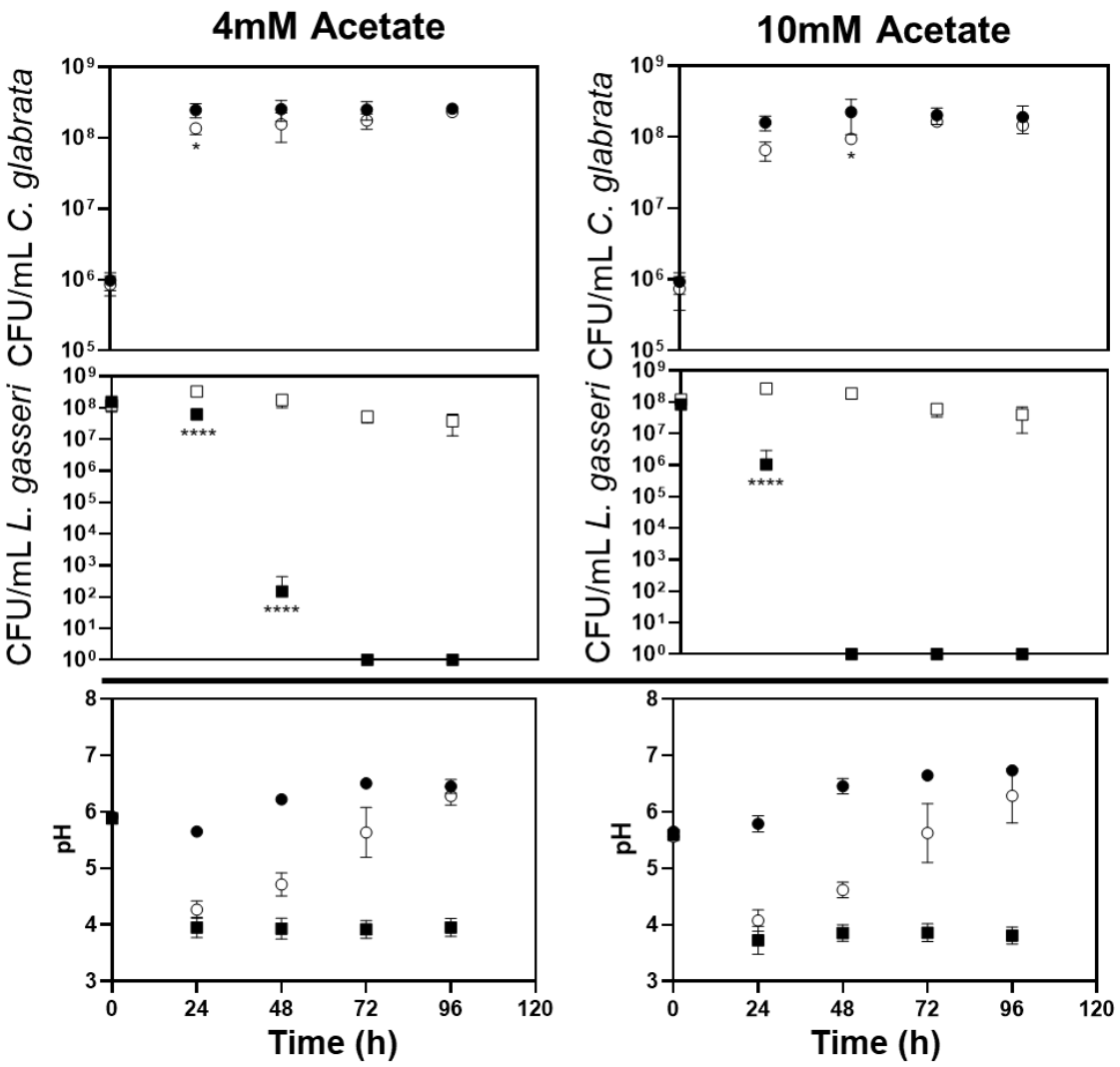
Annex Figure II.4. (A) Quantification of glucose (●), lactic acid (LA) (○) and acetic acid (AA) (■) in supernatants of cultures obtained upon cultivation of *L. gasseri*, alone or in combination with *C. glabrata* or *C. albicans*, in MRS medium. **(B**) Quantification of glucose (●), lactic acid (LA) (○) and acetic acid (AA) (■) in supernatants of cultures obtained upon cultivation of *L. gasseri*, alone or in combination with *C. glabrata* or *C. albicans*, in MRS-NaCl medium. **(C)** Quantification of glucose (●), lactic acid (LA) (○) and acetic acid (AA) (■) in supernatants of cultures obtained upon cultivation of *L. gasseri*, alone or in combination with *C. glabrata* or *C. albicans*, in MRS having 50 mM NaCl and 10 mM sodium acetate. The results obtained correspond to those obtained in three independent replicas.



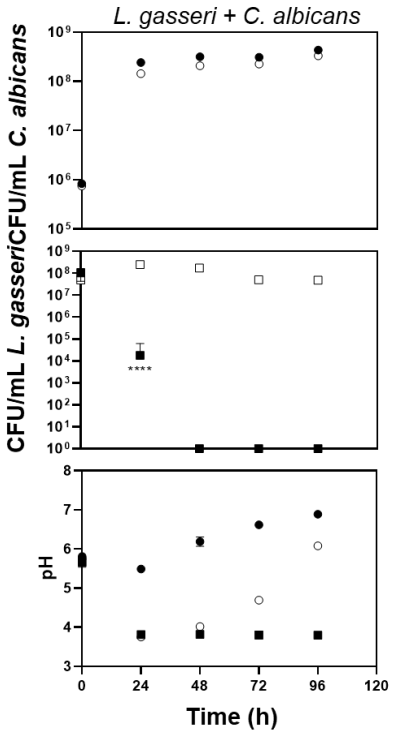
Annex Figure II.4. (B) Quantification of glucose (●), lactic acid (LA) (○) and acetic acid (AA) (■) in supernatants of cultures obtained upon cultivation of *L. gasseri*, alone or in combination with *C. glabrata* or *C. albicans*, in MRS-NaCl medium. The results obtained correspond to those obtained in three independent replicas.



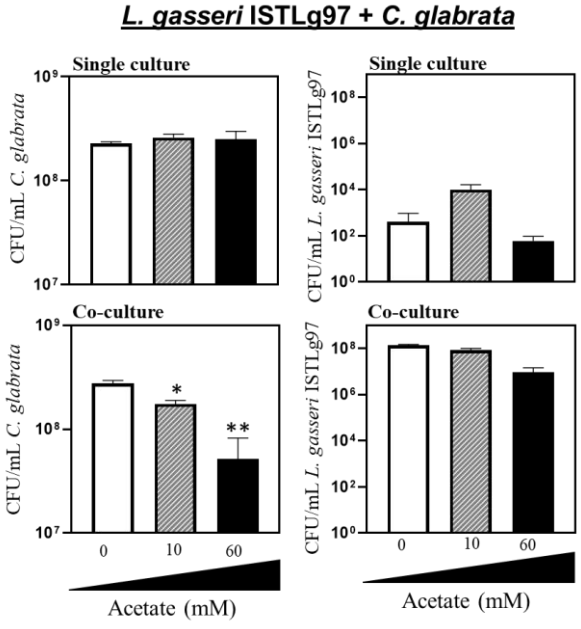
Annex Figure II.4. (C) Quantification of glucose (●), lactic acid (LA) (○) and acetic acid (AA) (■) in supernatants of cultures obtained upon cultivation of *L. gasseri*, alone or in combination with *C. glabrata*, in MRS having 50 mM NaCl and 10 mM sodium acetate. The results obtained correspond to those obtained in three independent replicas.



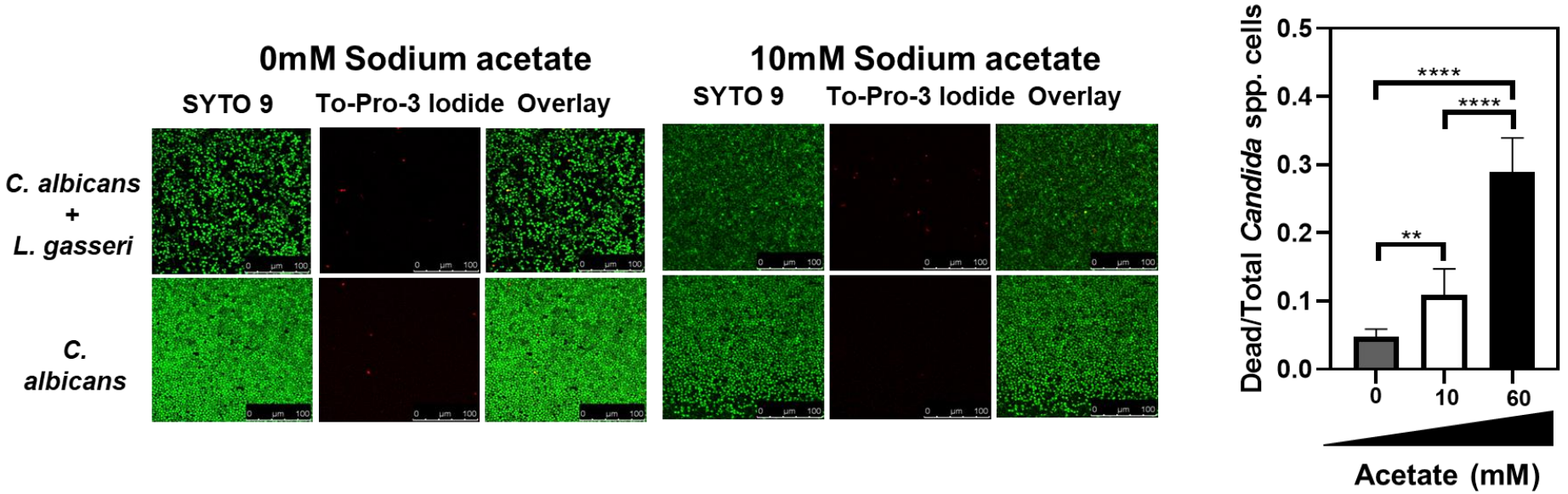
Annex Figure II.5. Cellular viability and medium pH during single or co-cultivation of *C. glabrata* (○,●) with *L. gasseri* (□,■) in MRS medium having 4 or 10 mM acetate (supplied in the form of sodium acetate). To maintain the total amount of sodium in 60 mM (the amount present in canonical MRS medium), it was further supplemented the MRS medium with 56 or 50 mM sodium chloride. The cells were cultivated, alone or in the presence of each other, in the same conditions described in Figure II.1. Filled symbols correspond to the samples taken during single-species cultivation while open symbols correspond to the samples taken during co-cultivation. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).



Annex Figure II.6. Cellular viability and medium pH during single or co-cultivation of *C. albicans* (○,●) with *L. gasseri* (□,■) in MRS medium having 60 mM sodium chloride (instead of the 60 mM sodium acetate present in canonical MRS medium). The cells were cultivated, alone or in the presence of each other, in the same conditions described in Figure II.1. Filled symbols correspond to the samples taken during single-species cultivation while open symbols corresponds to the samples taken during co-cultivation.; Statistical significance of the differences found in the presence or absence of acetate was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).



Annex Figure II.7. Viability of *C. glabrata* after 96h of single or co-cultivation with the vaginal clinical *L. gasseri* LgIST97 in MRS medium (corresponding to a concentration of acetate of 60 mM) or in this same medium having 60 mM NaCl (in replacement of the sodium acetate) or 10 mM sodium acetate and 50 mM NaCl. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).



Annex Figure II.8. Live/dead imaging of single-species or mixed biofilms formed by *L. gasseri* and *C. albicans* after 24h of cultivation, at 37oC and 25 rpm, in MRS without acetate (having instead 60 mM sodium chloride) or having 10 mM sodium acetate. The chart on the right corresponds to the quantification of dead *Candida* cells in the single-species or in the multi-species biofilms formed, based on quantification of the number of red-labelled yeast cells in all pictures taken from the biofilms, compared to the total number of *Candida* cells in the field (corresponding to green-labelled cells). *C. albicans* single and mixed biofilms scale bars correspond to 100 μm. For this quantitative analysis more than 1000 yeast cells were imaged in each condition. The data shown obtained with 60 mM acetate is the same shown in Figure II.3. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001).

Annex Table III.1. Summary of the mapping results for each sample. Total reads, rRNA depletion and reads mapped (including once or multiple times) of *C. glabrata* (Cg) and *L. gasseri* (Lg) single and co-culture after 2 and 8 hours of growth at 37ºC and 100 rpm are described.

Sample ID	Total Reads	Reads mapped in the genome of Cg	Reads mapped in only one locus of the Cg genome	Reads mapped in the genome of Lg	Reads mapped in only one locus of the Lg genome	Reads mapped to rRNA
Cg (single 2h)	23592891	22719594 (96.30%)	21915729 (92.89%)	-	-	6134151 (26.00%)
Cg (single 8h)	31787714	30762596 (96.78%)	30000220 (94.38%)	-	-	13710041 (43.13%)
Lg (single 2h)	21621757	-	-	21188429 (98.00%)	21060884 (97.41%)	0 (0.00%)
Lg (single 8h)	25764149	-	-	25044104 (97.21%)	24920170 (96.72%)	180349 (0.07%)
Co-culture (2h)	32156887	17832237 (55.45%)	17395994 (54.10%)	13497661 (41.97%)	13410945 (41.70%)	8402594 (26.13%)
Co-culture (8h)	25877569	19606239 (75.77%)	19089115 (73.77%)	5444188 (21.04%)	5422593 (20.95%)	9075263 (35.07%)

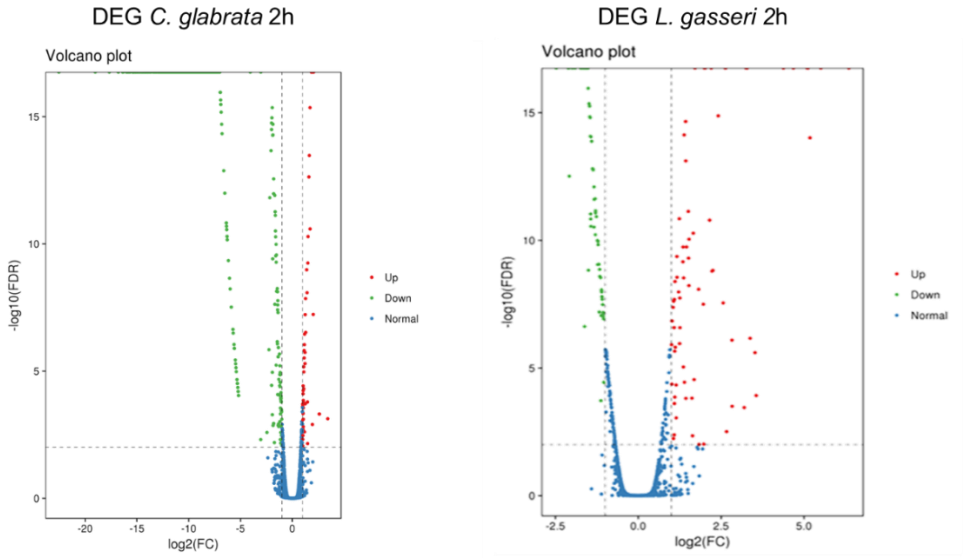
Annex Table III.2. Potential new transcripts of *L. gasseri*. Transcripts were identified manually by the presence of reads in genomic regions without a described annotation. Analysis was performed using CLC Genomics Workbench 10.1.1. Blastx analysis was performed in NCBI. https://docs.google.com/spreadsheets/d/16L4Br1NNUfZqgxUprHr_e87bcMWKrEj9R2v8Ij8WowY/edit?usp=sharing

Annex Table III.3. All novel intergenic/anti-sense transcripts of *C. glabrata* and *L. gasseri* identified in the RNA sequencing data and the novel intergenic/anti-sense transcripts that were differentially expressed in co-cultivation. https://docs.google.com/spreadsheets/d/1Slp3RBU62PCBzo5AF1kylx8csMsT9tc-QL7JSCEV3m8/edit?usp=sharing. Transcripts of *C. glabrata* previously identified as possible long non-coding RNA were identified using the database CandidaMine v0.1.beta (https://*Candida*mine.org/*Candida*mine/begin.do) and highlighted in grey.

Annex Table III.4. DEGs of *C. glabrata* and *L. gasseri* after 2 and 8 hours of growth. Table shows the systematic name and gene name when available, *S. cerevisiae* ortholog, log2FC, FDR and the description based on the *Candida* Genome Database (CGD) of each gene of *C. glabrata*, while *L. gasseri* tables show the gene ID in two different nomenclatures, gene description based on the NCBI, log2FC and the FDR of each gene. https://docs.google.com/spreadsheets/d/1m6KmgFOvG92DARZkZkP0-FPxKW4YJMuCp2-1L6MeSlQ/edit?usp=sharing

Annex Table III.5. Common *C. glabrata* and *L. gasseri* DEGs after 2 and 8 hours of growth. Note that some genes were up and down-regulated at both time points, while some showed a shift from up-regulation to down-regulation after 8 hours or vice-versa. The table describes the gene ORF, the fold change at 2 and 8 hours of co-cultivation, COG annotation and the description of each gene. For *C. glabrata* was further added the information regarding the gene name and *S. cerevisiae* ortholog. These DEGs were used to construct the graph shown in Figure III.5, https://docs.google.com/spreadsheets/d/1T-x57GrbsboPG68wp-2FqXlil8f0lmM6IMbYHvLgOJk/edit?usp=sharing.

Annex Table III.6. COG annotation of DEGs of *C. glabrata* and *L. gasseri* used to construct the graph shown in Figure III.6. Gene ID and COG annotation are described for each time point and up and down-regulated genes of *C. glabrata* and *L. gasseri,* https://docs.google.com/spreadsheets/d/14VQ4bsjqix16I-OdUs_oH3_OvdiedyFoX_jsqyeDbmc/edit?usp=sharing.



Annex Figure III.1. Volcano plots of the differentially expressed genes (log2(FC)) of *C. glabrata* and *L. gasseri* after 2h of growth in MRS at 37ºC and 100 rpm. Each dot represents a single gene and the red dots are the up-regulated genes while the green dots are the down-regulated genes in co-cultivation. Blue dots were not significantly differentially expressed in co-cultivation settings.

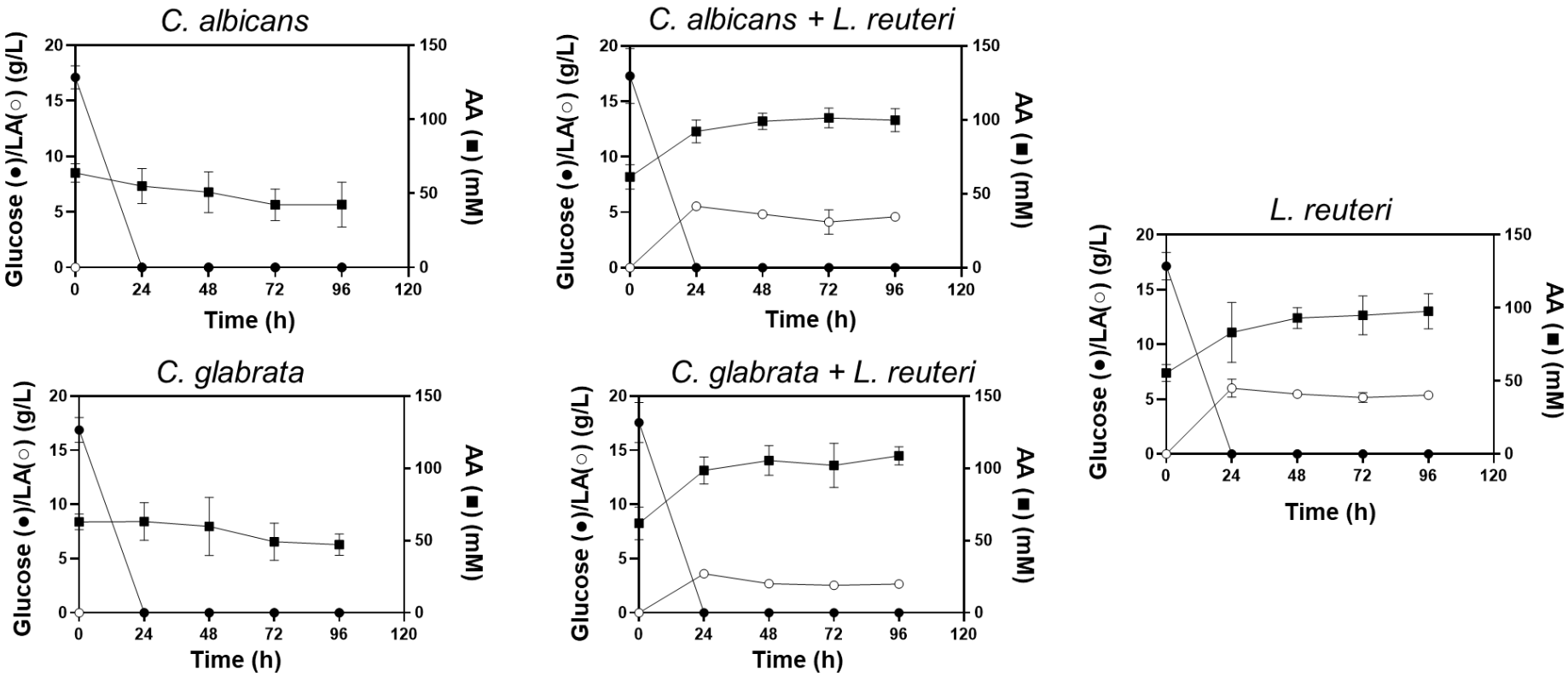
Annex Table IV.1. List of *C. glabrata* deletion strains in the mutant collection. Table shows the ORF deleted, gene name when available, the background strain and the description of the ORF based on the website *Candida* genome database (http://www.candidagenome.org/). Note that some *C. glabrata* deletion strains were built in both background recipient strains (HTL and Δ*his3*), https://docs.google.com/spreadsheets/d/1S8aDqVSsuJtKmwjRaCK7XnZRxuWcHzqBReZJ35V8GUY/edit?usp=sharing

Annex Table IV.2. List of the upstream and downstream barcodes of the *C. glabrata* mutants comprising the collection. Table described the ORF, the background strains used to construct the deletion strains and the respective upstream and downstream barcode, https://docs.google.com/spreadsheets/d/1hC479dDdPrt-IG0w_BRxiKV-WLKTjB8laS8l_aG01kc/edit?usp=sharing

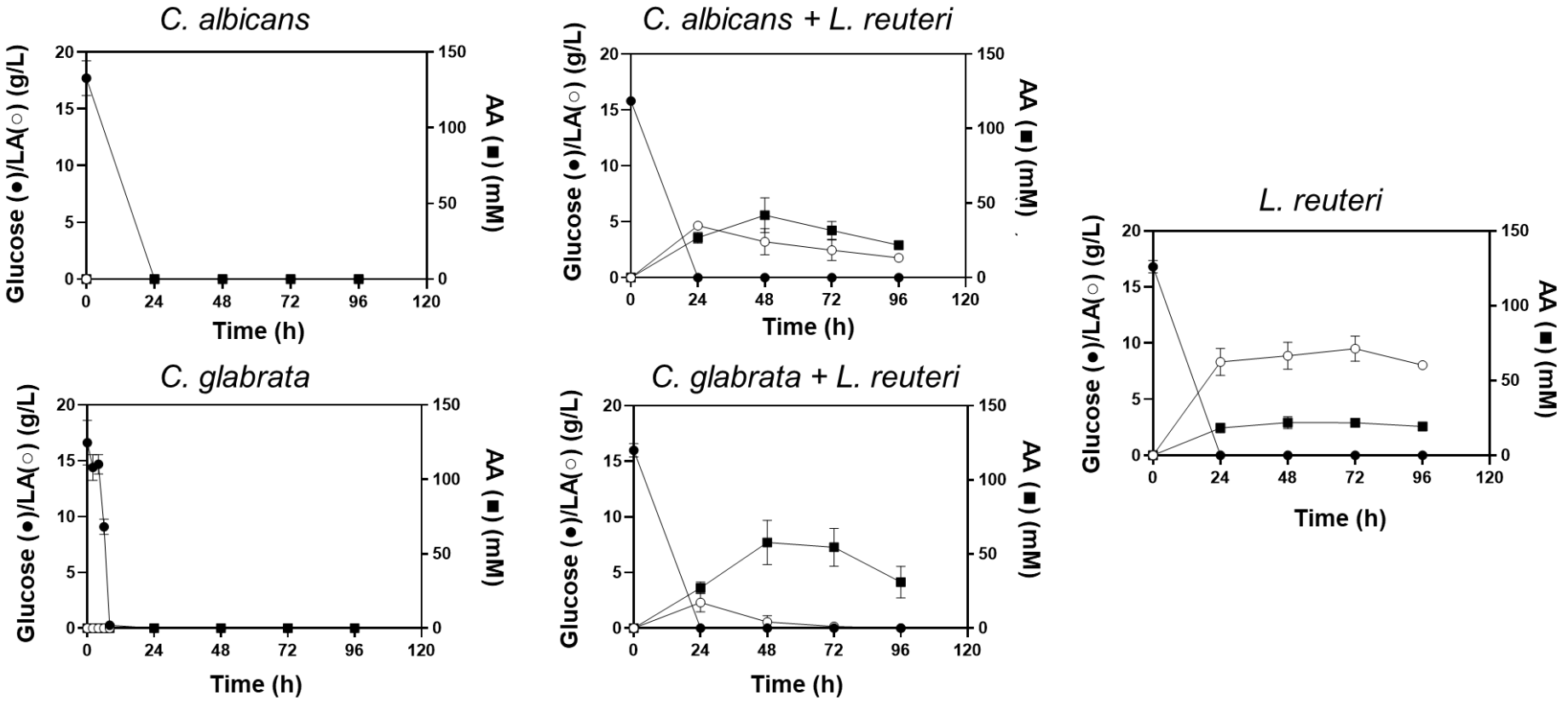
Annex Table IV.3. List of *C. glabrata* mutant strains not phenotyped because of wrong associations between barcodes and strain identification which resulted in the pooling of strains having similar barcodes in the 96 mutant pools that were constructed, https://docs.google.com/spreadsheets/d/1BBRYVXu7svgr86yifDRKpcOwzrJ6MKeUSJPUL9Dup_s/edit?usp=sharing

Annex Table IV.4. List of *C. glabrata* mutant strains with enhanced or poor growth compared with the wild-type in pooled growth in single cultivation. The threshold used for the considerably less fit strains was a ratio under 0.3 (mutant/WT) while for the strains with increased fitness was a 1.5 ratio (mutant/WT) as a threshold, https://docs.google.com/spreadsheets/d/1ZZ0gH69h4bnvRZMHJ28oAYYGSCzklx6oNDdwiR9A_4A/edit?usp=sharing

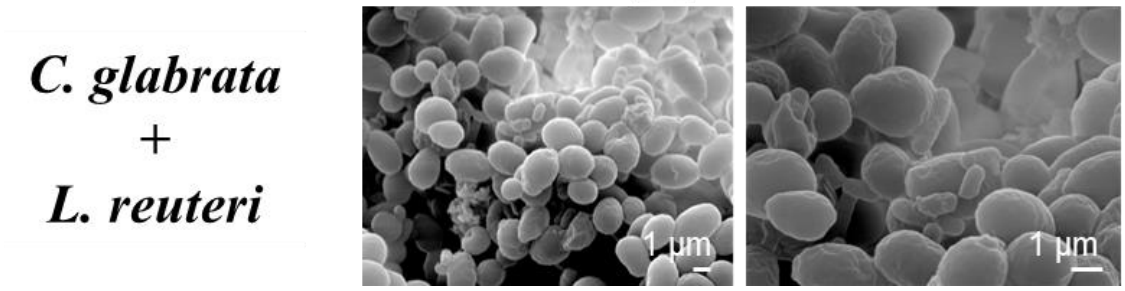
Annex Table IV.5. List of *C. glabrata* deletion strains susceptible to the presence of *L. gasseri* in biofilm formation conditions. Growth was performed at 37ºC and 25 rpm for 24h in MRS. Competitiveness was determined based on PI fluorescence and compared with the WT strain. Susceptible strains were identified based on two aspects: 1st only deletion strains with similar PI fluorescence with the WT in single cultivation were considered; 2nd was identified the strains with enhanced PI fluorescence compared with the WT in co-cultivation (at least 2.5-fold). Susceptible strains were determined using statistical significance with one-way ANOVA. Table described the ORF, the background strain, the gene name when available, the S. cerevisiae ortholog, the decreased viability (fold change), the biological function and a brief gene description. https://docs.google.com/spreadsheets/d/1pAlNiwz5LODTrSvaXN56n1LCoOSzi118qnpFtXzfzUc/edit?usp=sharing



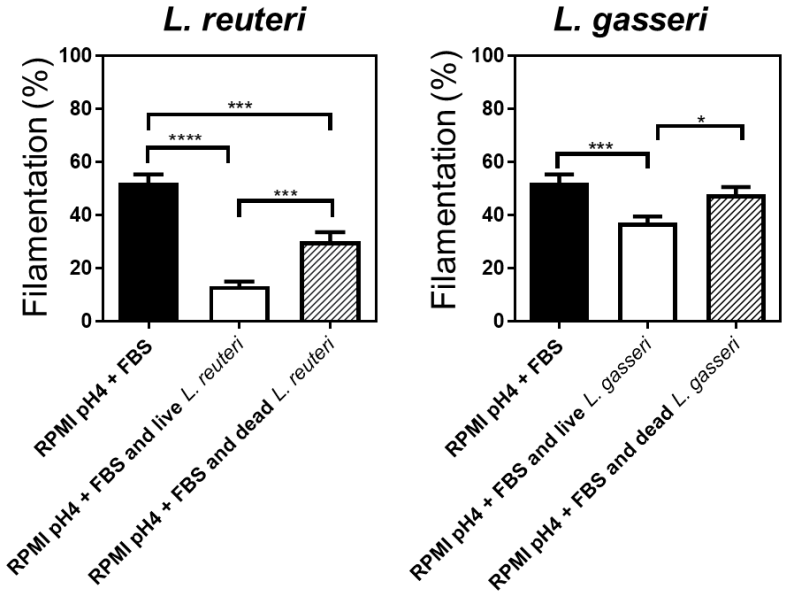
Annex Figure V.1. Quantification of glucose (●), lactic acid (LA) (○) and acetic acid (AA) (■) in supernatants of cultures obtained upon cultivation of *L. reuteri*, alone or in combination with *C. glabrata* or *C. albicans*, in MRS medium. The results obtained correspond to those obtained in three independent replicas.



Annex Figure V.2. Quantification of glucose (●), lactic acid (LA) (○) and acetic acid (AA) (■) in supernatants of cultures obtained upon cultivation of *L. reuteri*, alone or in combination with *C. glabrata* or *C. albicans*, in MRS-NaCl medium. The results obtained correspond to those obtained in three independent replicas.



Annex Figure V.3. SEM images of *L. reuteri* and *C. glabrata* mixed biofilms. The biofilms were obtained using the same conditions described in materials and methods, with the exception that was used 5 mL-polystyrene plates instead of the 8mm plates used in the fluorescence microscopy imaging. These images were obtained after 24h of cultivation at 37ºC and 25 rpm using a magnification of 5000x to 10000x over the dried biofilms.



Annex Figure V.4. Filamentation ratio of *C. albicans* in the presence of *L. reuteri* and *L. gasseri* live and dead cells. Growth was performed at 37ºC at 100 rpm for 24h in RPMI pH 4 supplemented with 10% FBS to induce filamentation. This data was obtained from a large set of images taken using an optical microscope with a magnification of 1000x. Statistical significance was calculated using one-way ANOVA (*p-value below 0.1; **p-value below 0.01; ***p-value below 0.001; ****p-value below 0.0001). Data was obtained from three independent replicates.