Unveiling the molecular basis of *S. boulardii* probiotic activity: through the construction of a global promoter survey computational tool and transcriptomics analyses

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Thesis to obtain a Master's Degree in:

Biological Engineering

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October 2019

Preface

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences of Instituto superior Técnico and INESC-ID (Lisbon, Portugal), during the period February-October 2019, under the supervision of Professor Miguel Nobre Parreira Cacho Teixeira and Professor Pedro Tiago Gonçalves Monteiro.

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

This document was written and made publicly available as an institutional academic requirement and as a part of the evaluation of the MSc thesis in Biological Engineering of the author at Instituto Superior Técnico. The work described herein was performed at the Institute for Bioengineering and Biosciences of Instituto superior Técnico (Lisbon, Portugal), during the period February-October 2019, under the supervision of Professor Miguel Nobre Parreira Cacho Teixeira and Professor Pedro Tiago Gonçalves Monteiro.

This work was financially supported by “Fundação para a Ciência e a Tecnologia” (FCT) (Contract PTDC/BII-BIO/28216/2017) and is also integrated in the project LISBOA-01-0145-FEDER-022231 – the Biodata.pt Research Infrastructure, supported by Lisboa Portugal Regional Operational Programme (Lisboa 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). Funding received by Instituto de Bioengenharia e Biociências (iBB) from FCT (grant UID/BIO/04565/2013) and Programa Operacional Regional de Lisboa 2020 (project no. 007317) is also acknowledged.

I would like to express my utmost gratitude to Professors Miguel Nobre Parreira Cacho Teixeira and Pedro Tiago Gonçalves Monteiro for all the guidance, support, availability and patience they offered me. They both went over and beyond and gave their best in their role as advisors.

I would also like to thank Doctor Pedro Pais for all the detailed and patient guidance in the laboratory, for helping me not to panic with unexpected results and for the extra support throughout the entire thesis.

For letting me take my own pace, creating the best studying conditions and always supporting me and raising my morale, I would like to specially thank my mother, Maria Vanda da Costa Pinto de Almeida Sá Dias. For the ever present (though far away) brother, Mário Manuel Sá Dias Pinto de Almeida, I would like to thank all the late night skype lessons and doubt clarifications about programming, even on busy days.

For the ever constant mental and moral support, without which I would not have been able to reach this point, I would like to specially thank my boyfriend, Tiago Miguel Garcês dos Santos Filipe, who has been with me through every crisis.

I would also like to thank my best friends, Sara Calado, Susana Pereira and Diogo Carvalho for all their moral support through anything and everything.

Abstract

S. boulardii has shown very promising probiotic properties in most diseases that lead to gut dysbiosis. Although *S. cerevisiae* and *S. boulardii* share 95% homology, only *S. boulardii* seems to be probiotic, which is puzzling. A better understanding of the underlying properties is crucial for the optimization of probiotic strain selection and usage.

In this thesis, we have grown *S. boulardii* and *S. cerevisiae* in an intestinal like medium (ILM) and performed a transcriptional analysis in order to understand the different survival and probiotic characteristics that lead these two very similar species to display different phenotypic outcomes. It was concluded that *S. boulardii* appears to be more sensitive to salt stress, but to be better adapted to human body temperature and oxidative stress. *S. boulardii* was found to display up-regulation of genes associated with probiotic activity, when compared with *S. cerevisiae*, including some involved in polyamine (spermidine) and acetate biosynthesis, as well as cell wall and adhesion proteins. Consistently, *S. boulardii* was confirmed to display higher adherence to intestinal epithelium than *S. cerevisiae* in adhesion assays.

Additionally, a new functionality was implemented in the ProBioYeastract platform, to enable a global evaluation of promoter regions in *S. boulardii* genes, when compared to *S. cerevisiae* homologs. This tool was used to predict the regulatory changes that underlie the observed transcriptomic variation between *S. boulardii* and *S. cerevisiae*, pointing out the transcription factors Yap3 and Gcn4 as displaying a particularly different set of regulated genes in the two species, with a potential impact in intestinal tract adaptation.

Resumo

S. boulardii é promissora, relativamente às suas propriedades probióticas, para a maioria das doenças que causam disbiose intestinal. Embora *S. cerevisiae* e *S. boulardii* partilhem 95% de homologia, apenas *S. boulardii* é probiótica. Uma melhor compreensão das propriedades subjacentes é crucial para a optimização da seleção e uso da estirpe probiótica.

Nesta tese, *S. boulardii* e *S. cerevisiae* foram cultivadas num meio simulador do ambiente intestinal (ILM). Depois foi feita uma análise transcriptómica para compreender as diferenças de sobrevivência e de propriedades probtióticas que levam as duas estirpes a terem fenótipos tão diferentes. Conclui-se que *S. boulardii* é susceptível a stress salino, em especial a sais biliares, mas melhor adaptada à temperatura do corpo humano e ao stress oxidativo. *S. boulardii* mostrou ter maior expressão de genes associados a actividade probióticas, incluindo genes envolvidos na síntese de poliaminas (espermidina) e de ácido acético, tal como de genes associados à parede celular e proteínas adesão. Consistentemente, *S. boulardii* mostrou ter maior aderência ao epitélio intestinal do que *S. cerevisiae* em ensaios de adesão.

Além disso, uma nova funcionalidade foi implementada para a plataforma ProBioYeastract para permitir uma avaliação global de regiões promotoras em genes de *S. boulardii* quando comparados com homólogos de *S. cerevisiae*. Esta ferramenta foi capaz de prever alterações regulatórias que estão na base de diferenças transcriptómicas entre as duas estirpes, assinalando os fatores de transcrição Yap3p e Gcn4p como tendo uma conjunto de genes regulados particularmente diferentes nas duas estirpes e, portanto, com um potencial impacto na adaptação ao trato intestinal.

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List of abbreviations

AAD: Antibiotic-associated diarrhea

Akt: Anti-apoptotic protein kinase B

ANI: Average Nucleotide Identity

ARCOL: Artificial colon model

BCFA: Branched-chain fatty acid

CFU: Colony Forming Units

CGD: Saccharomyces Genome Database

CRT: Controlled and randomized trials

CT: Cholera toxin

EF: Edema factor

EFSA: European Food Safety Authority

EGF: Epidermal growth factor

ERDF: European Regional Development Fund

ET: Edema toxin

FBS: Fetal bovine serum

FCT: Fundação para a Ciência e a Tecnologia

HO: Homothallic switching endonuclease

HOG MAPK: High osmolarity glycerol mitogen activated protein kinase

HSR: Heat shock response

iBB: Instituto de Bioengenharia e Biociências

IFN-γ: Interferon-γ

ILM: Intestinal like medium

ITS: Internal transcribed spacer

LF: Lethal factor

LT: Lethal toxin

MAPK: Mitogen activated protein kinases

MDR: Multidrug resistance pumps

MRS: De Man, Rogosa and Sharpe agar

NF-Kb: Nuclear factor κB

NTS: non-transcribed spacer

PA: Protective-antigen

PFGE: Pulse-field gel electrophoresis

PPAR-γ: Peroxisome proliferator activated receptor-γ

RAPD-PCR: Randomly amplified polymorphic DNA-polymerase chain reaction

RFLP: Restriction fragment length polymorphisms

SCFA: Short chain fatty acids

SDA: Sabouraud (Dextrose) Agar

SP: Species

ST: Salmonella enterica serovar typhimurium

STRE: Stress response elements

TF: Transcription factor

TFBS: Transcription factor's binding sites

US FDA: US Food and Drug Administration

Yeastract+: Yeast Search for Transcriptional Regulators And Consensus Tracking +

Introduction

Objectives and thesis outline

Saccharomyces boulardii is a widely used probiotic. Nonetheless, the molecular basis of its mode of action is scarcely understood. Recently, the genome sequence of five commercial *S. boulardii* strains was obtained and compared to that of *S. cerevisiae* lab strains. But the very few observed differences could not be clearly correlated to the unique role of *S. boulardii*. [1,2,3,4,5]

In a previous study from our lab, a preliminary version of the ProbioYeastract database was built, gathering information on the genome sequence of two *S. boulardii* strains, namely Unique28 and Biocodex, at the level of gene and promoter sequences, as well as orthology predictions in comparison to the S288C *S. cerevisiae* reference strain. The analysis of a few promoter sequences, found up-stream of genes whose function was predicted to be associated to *S. boulardii* probiotic activity, suggested that differential gene expression levels could underlie the different phenotypes exhibited by *S. boulardii* when compared to *S. cerevisiae*.

In this thesis, we aim to test this hypothesis by performing global gene expression analysis, resorting to Illumina-based RNA-sequencing and to the development of novel computational tools for global promoter sequence analysis.

This thesis starts with an Introduction section that provides an overview of the current knowledge on the probiotic activity of *S. boulardii*, including a summary of the main biological processes that may underlie this activity *in vivo*.

Following, Materials and Methods are defined, in the order of their use in the production of the obtained results.

In the Results and Discussion section, the work starts with the definition of a growth medium that mimics the gastrointestinal tract environment, while supporting growth by both *S. cerevisiae* BY4741 and *S. boulardii* biocodex strains. RNA-seq is then used to study the transcriptome-wide differences among the two strains in an *in vitro* gastrointestinal tract-like environment, with emphasis on probiotic-related genes and functions. Simultaneously, bioinformatic tools for the comparison of inter- and intra-species genomic sequences is developed, aiming the automatic analysis of changes in gene promoter regions associated to specific changes in transcriptional regulation.

Finally, a section on Conclusions and Perspectives summarizes the outcomes of this work, highlighting its contribution to the advancement of knowledge. Additionally, it provides guidelines for future work in the field, which are believed to be useful to guide the design of more effective therapeutic approaches.

Probiotic definition

Probiotics are defined as live organisms which, when administered in adequate amounts, confer a health benefit to the host, independently of where the action takes place and of the type of administration. They are normally recommended to help strengthen the host and assist in the recovery of certain diseases. According to this definition, probiotics in food must contain at least 10^6 CFU/g of viable and active microorganisms, while freeze-dried supplements have shown good results with 10^7 to 10^{11} viable microorganisms per day. [1,2,3,4,5] It is also preferable that these are of human origin and that they cannot transfer any antibiotic resistance, pathogenicity or toxicity factors. [4]

Besides probiotics, there are also prebiotics, symbiotics and postbiotics. The first are non-digestible food ingredients that benefit the host by selectively stimulating the growth or activity of one or of a limited number of microorganisms in the colon. For example, the ingestion of substances like fructooligosaccharides, inulin, trans-galactosylated oligosaccharides and soybeans' oligosaccharides promote the growth of bifidobacterium. In order to be considered as prebiotics, the ingredients must not be hydrolyzed nor absorbed in the upper gastrointestinal tract, must have the ability to change colonic flora to a better composition and must promote luminal or systemic beneficial effects to the host.[6] Symbiotics or eubiotics are a product that contains a probiotic and a prebiotic that selectively favors that specific probiotic by helping it survive ingestion and colonize the intestinal tract. [2,4,6] Postbiotics are soluble components with biological activity, which can consist of metabolic by-products, dead microorganisms or non-viable microbial products with probiotics' properties. The last can be a safer alternative to the use of viable microorganisms. [4]

It should be noted that probiotics are considered food supplements in many countries, since the capacity to prevent and treat diseases has not been attributed to them yet. Even though there are several requests for this attribution, the European Food Safety Authority (EFSA) and US Food and Drug Administration (FDA) have not accepted any of them, normally for the same reasons: insufficient characterization, undefined or non-beneficial claims, lack of relevant human studies and lack of studies with good quality. [4]

The most commonly used probiotics in clinical procedures are usually lactic acid producing bacteria (*Lactobacillus*, *Bacillus*, *Bifidobacterium*, *Streptococcus* and *Enterococcus* species and *Lactococcus lactis*). This heterogeneous group of microorganisms is normally found in the human gastrointestinal tract. Its introduction in the system is done by ingestion of fermented food, like yogurt, kefir, miso, among others. [4]

In food supplements, these probiotics can be used by themselves or combined with each other: *Lactobacillus* with *Bifidobacterium*, *Enterobacterium* or *Bacillus*. It should be noted that not all combinations are stable and can perform their purpose.[7] Different strains of the same probiotic bacteria can have effects based in different capabilities or enzymatic activities, even if they belong to the same species. Besides that, different microorganisms have different preferences for different habitats, which can change with the host. We can consider that there are 4 different microhabitats in

the gastrointestinal system: the epithelial cells' surface; ileum's, cecum's and colon's crypts; the mucous gel that covers the epithelial and the lumen. Several indigenous probiotics and pathogens adhere specifically to the epithelial cells' surface by, for example, mediation of special organelles, like fimbriae. The crypt is normally colonized by bacteria with spiral and motile form, like *Borellia*, *Treponema*, *Spirillium*, *Helicobacter pylori*, among others. The mucous layer that covers the epithelium can protect the host against colonization in certain situations. The microflora in the lumen varies immensely with the intestinal traffic: the lower intestine density is low when compared with the colon, where there is an abundant quantity of microorganisms that do not need adhesion molecules. For example, *Lactobacilli* colonizes the human lower ileum, where there is a lot of traffic, so the bacteria need to adhere strongly to the mucous epithelium and adapt to the milieu of this adhesion site. This means that the competition between probiotic microorganisms and pathogens is dependent on these habitat-related issues.[2]

Probiotics are available in two main forms: supplements and fermented food. Supplements can be found in various forms too: lyophilized culture capsules, powder or tablets; capsules with a mixture of several probiotics. Fermented foods are mixtures of lactic products and food products (like drinks, kefir, chocolate, wafers, sauerkraut…).[5] They can also be administered in different ways, for example, orally, subcutaneously, among others.[1] The advantages and disadvantages of each form can be seen in *Table 1*. We should also consider if, when administered orally, probiotics are ingested while fasting (with only a cup of water) or with a meal, since food carriers have specific physochemical properties as, for example, buffering capacity, water activity, redox potential, protein content, sugar content, pH and temperature.[8,9] It is important to note that probiotics may also be negatively affected by some foods and medications such as alcohol and antibiotics.[5]

However, it is not only bacteria that possess probiotic properties, yeasts may also exhibit these characteristics. *Saccharomyces cerevisiae* has been poorly studied in relation to its probiotics properties. [1] Even though its normal variant does not seem to have these characteristics, it is known that the *boulardii* variant does have and is effective in the treatment of acute and chronic intestinal diseases. To date this is the only yeast used as a probiotic.[4]

According to several studies, many probiotics are incorrectly labeled, since they contain contaminants, they do not include the referred strain or they include it but in very different (lower) concentrations. In 14 American commercialized probiotics, 93% were incorrectly labeled: 57% had contaminants, 36% didn't include the listed strains and 50% had doses lower then referred. In another study, from 6 probiotics only half had the amounts they were supposed to. These 3 were produced by Merck, NeoChemical e Herald's. In yet another study, from 15 Belgian probiotics, 13 had the referred concentration with a variation of one log at most. This shows that, before choosing a probiotic, it is essential to verify which company produces it and if that company sponsors clinical trials, since that reveals a good degree of compromise. A good example is the *S. boulardii* CNCM I-745 (or *S. boulardii* Hansen CBS 5926) strain produced by Laboratoires Biocodex in France which is supported by more than 88 controlled and randomized trials. Nevertheless, the efficacy of this strain cannot be extrapolated to other strains, like *S. boulardii* CNCM 1079. [1]

Table 1: Advantages and disadvantages of different probiotic's ingestion methods. Adapted from Arain et al [5]

Probiotic's ingestion method	Advantages	Disadvantages
Capsules with lyophilized culture	• Easy oral administration • Contains no binders • May be incorporated into food or beverages	• Not usable for the upper gastrointestinal tract, unless opened • May contain allergenic excipients • Higher cost • When opened, *S. boulardii* may become airborne and cause contaminations
Powder with lyophilized culture	• Effective in the upper gastrointestinal tract • Easy to adjust doses • May be incorporated into food or beverages • Contains no binders	• *S. boulardii* may become airborne and cause contaminations
Tablets with lyophilized culture	• Easy oral administration • Effective in the upper gastrointestinal tract	• May contain allergenic or problematic excipients or binders • Higher cost
Fermented diary	• Cheap • Good availability • Easy to integrate into daily habits • Additional nutritional benefits • Enhanced bacterial survival through the upper gastrointestinal tract: 100x lower viable bacteria needed per dose to achieve the same amount of viable bacteria in the lower bowel • Effective in the upper gastrointestinal tract	• Contains dairy proteins and lactose • Has a specific taste • Not suitable for travelling • Not suitable for vegans

Extrapolation of probiotic's properties observed *in vitro* and animal's studies to human trials is very difficult, since it would essentially consist in comparing similar effects by a specific strain but in different contexts. The differences found between hosts of the same species are in part due to specific host characteristics (such as genetic factors, baseline immune functions, microbiome diversity, different body sites targeted, intra-person variation, between other) which can vary from person to person and environmental factor (such as diet, stress, in between others) that can be partially adjusted by the host.[4]

To complicate matters further, probiotics properties hugely differ between different or similar species, strains or even between different (but still very similar) strain variants, which means these properties are strain variant-specific. Hence, in commercial products, studies of health benefits and adverse reactions should be done on the specific strain being sold. Furthermore, extrapolating both positive and negative effects of a certain strain to others and making meta-analysis of these effects using different active molecules should be avoided. Interpretation of data from studies is complicated, since the use of different strains, dosages, duration of treatments and size of trials all play a role. It is

not yet known if there exists optimal probiotics species and doses for certain diseases and head-to-head strain's comparison should be done in order to help understand results better.[4]

S. boulardii versus *S. cerevisiae*

Saccharomyces boulardii was discovered by Henri Boulard in 1920. It was obtained from tea made with litchi and mangosteen's peel. In 1947, the Laboratories Biocodex created a patent for this yeast with which research and manufacturing protocols were made. Normally this probiotic is prepared through lyophylization of live yeast and encapsulation with a preparation of lactose. Nowadays, this yeast is used in more than 80 countries in Europe, North and South America, Asia and Middle East. Even though it was initially called *S. boulardii*, hence a different species when compared to *S. cerevisiae*, it is still debatable, though somewhat accepted, if it should not be considered a variant of *S. cerevisiae* instead. Although they are, indeed, very close genetically (95% DNA homology) and have a very similar karyotype. *S. boulardii* is physiologically and metabolically different from *S. cerevisiae* (*Table 2*). For example, it is incapable of producing ascospores or switching to haploid form, assimilating galactose as a carbon source and of having α-glucosidase activity. It also has dissimilar oxidative utilization and fermentation patterns. It is more resistant to temperature and acidic stresses, but less resistant to bile salts. However, phenotypic characteristics such as these cannot be used for identification. [1,5,10,11]

Table 2: Characteristics of S. cerevisiae and S. boulardii. [1,5,12,11,13]

Characteristics		***S. cerevisiae***	***S. boulardii***
Optimal growth temperature		30 a 33 ºC	37 ºC
High temperature resistance (52ºC)		45% viability	65% viability
Acid pH resistance (pH=2 for one hour)		No (30% viability)	Yes (75% viability)
Tolerance to bile acids (>0.3%(w/v))		No Only survives until 0.15%(w/v)	No Only survives until 0.10%(w/v)
Basic pH resistance (pH=8)		Yes (Similar viability)	Yes (Similar viability)
Assimilation of galactose and α-glucosidase activity		Yes	No
Ploidy		Diploid or haploid	Always diploid
Homo or heterothallic		Homothallic	Homothallic
Mating type		Both	Both
Sporulation		Sporogenous	Asporogenous, but produces fertile hybrids with *S. cerevisiae*
Pseudohyphal switching		Normal	Increased
Retrotransposon (Ty elements)			No intact Ty1, 3 or 4 elements
Adherence to epithelial cells	**Normal microbiota (mice and human)**	No	No
	Gnotobiotic mice	Unknown	Yes
	Humans treated with ampicillin	Unknown	Yes

It has been shown by pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), restriction fragment length polymorphisms (RFLP) of non-transcribed spacer (NTS) or internal transcribed spacer (ITS) that *S. boulardii* from different origins all belong to a clearly delimited cluster within *S. cerevisiae* species and, hence, constitute

different strains of the same species. The difference between this cluster and other isolates was only a 1640 kb band (chromosome 4), two distinct RAPD fingerprints.[14,11]

A study using microsatellites, short sequence repeats that have substantial polymorphism, found that (CAG)9 sequence (allele) at locus 4 is specific for *S. boulardii* and is not shared with different strains of *S. cerevisiae*.[10] One proposed way to differentiate the two and identify *S. boulardii* properly is to observe the microsatellite polymorphization of *YKL139W* and *YLR177W* genes and the hybridization of Ty917. [1,5,11]

A study used DNA/DNA hybridizations with spotted microarrays and full-length PCR products for all ORFs of *S. cerevisiae* (study I)[15]. Hence, all genes encoding proteins were analyzed. This analysis was done between *Saccharomyces* sensu stricto complex (*S. cerevisiae*, *S. paradoxus*, *S. cariocanus*, *S. mikatae*, *S. kudriavzevii*, *S. bayanus*, and *S. pastorianus*) or including *S. castellii*, *S. boulardii*, *S. cerevisiae* _1278b, a strain distant from the reference strain S288c and *S. cerevisiae* FY1679, which is a direct derivative of S288c. With this both intra and interspecific species were compared. This method was fully able to differentiate species within the *Saccharomyces* sensu stricto complex. It also showed that *S. cerevisiae*_1278b was the closest to *S. cerevisiae* FY1679, while *S. boulardii* was further away and, hence, more distantly related. However, *S. boulardii* was still closer to *S. cerevisiae* FY1679 than other species in the *Saccharomyces* sensu stricto complex, indicating that this yeast is a strain of *S. cerevisiae* and not a different species. In order to verify how much *S. boulardii* position in the phylogenetic tree depended on differences of Ty elements, ORFs associated to these elements were dismissed and the tree was redone. All species and strains maintained their location in the tree except for *S. boulardii* that moved significantly closer to *S. cerevisiae* FY1679, even more so than *S. cerevisiae*_1278b. This suggests that instead of being a different species, *S. boulardii* is a *S. cerevisiae* that lost all intact Ty1/2 elements.[15]

Another study (study II)[16] used Average Nucleotide Identity (ANI) and found that *S. boulardii* and *cerevisiae* are 99% similar in terms of genome sequence. This means that there is a big conservation in protein repertory between *S. boulardii* and *S. cerevisiae*. It is known that *S. boulardii* has antimicrobial activity by secreting 54, 63 and 120 kDA proteins that cleave microbial toxins or reduce cAMP levels. [1,16–19,20] However, no *S. boulardii* specific and unique proteins were found that could match these three proteins. From the core proteome, 182 proteins with orthologs in all strains were fetched and concatenated in order to create a phylogenetic tree (*Figure 1*). *S. cerevisiae* strains formed groups according to their isolation source, while *S. boulardii* strains formed a clade. *S. boulardii* unique28 was closest to *S. cerevisiae* UFMG A-905 (excluding other *S. boulardii* strains), which belonged to the same clade. It should be noted that *S. cerevisiae* UFMG A-905 strain is also probiotic. Interestingly, *S. boulardii* cluster was shared with *S. cerevisiae* wine (BC187, YJM1387, YJM1417, YJM1332, R008) and brewery strains isolated from fruits (YJM1477 and YJM1242), but wine strains were closer to *S. boulardii* than brewery strains. Laboratory strains formed separate clusters, while clinical isolates were grouped in three distant clusters. It can be easily concluded that *S. boulardii* is definitely a strain of the *S. cerevisiae* species. [16]

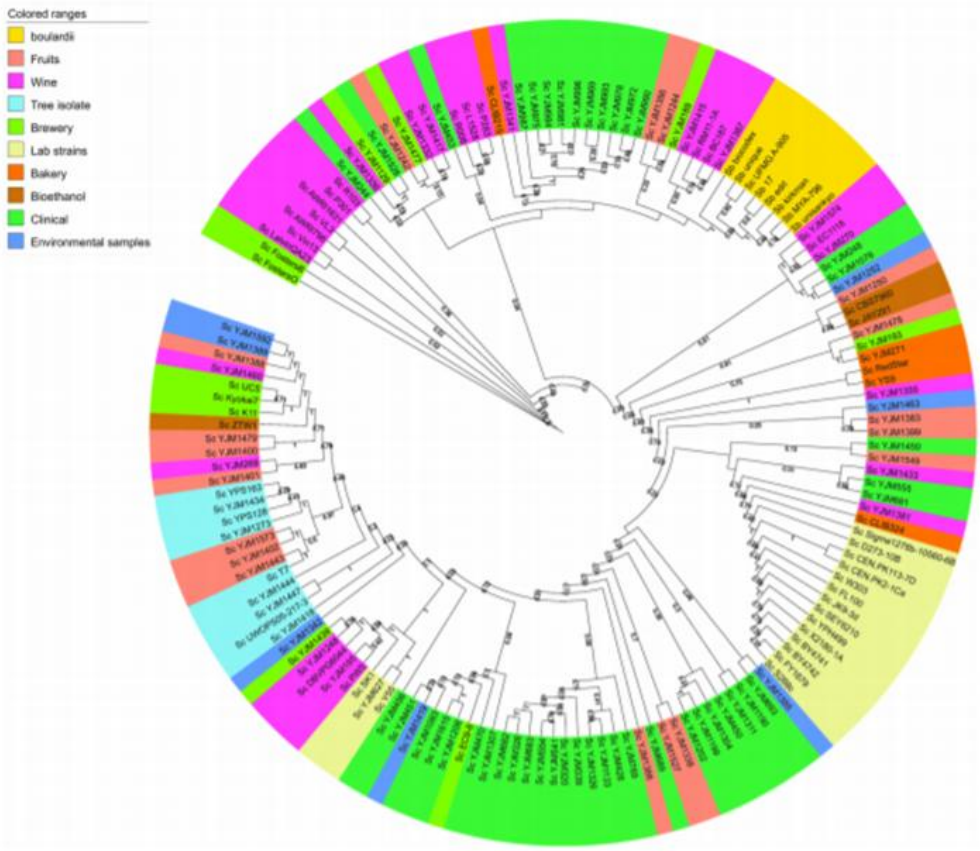


Figure 1:Phylogenetic tree from study II. [16]

Another study (study III)[21] used CGH with oligonucleotide microarrays and showed that *S. boulardii* UL, UL#3509 and UL[rho] are diploid strains with two copies of each chromosome except for chromosome IX, which has three copies. Hence, *S. boulardii* is an aneuploid. It has been shown that *S. cerevisiae* is able to modulate and maintain aneuploidy in individual chromosomes, probably in order to gain a selective advantage from extra copies of genes contained in the duplicated chromosome. This applies to *S. boulardii* chromosome IX too.[21] Study II sequenced the whole *S. boulardii* Biocodex and unique 28 genome and found that the first had 16 complete chromosomes and 14 unplaced contigs, while the second had 14 complete chromosomes, two chromosomes (V and IX) with two contigs and nine sole contigs. All *S. boulardii* strains had about 300 predicted tRNAs and 5140 proteins in the core proteome. Chromosome IX trisomy was not found, unlike the previous study, whereas chromosome XII had double read coverage when compared with other chromosomes, which may indicate aneuploidy.[16]

S. boulardii and *cerevisiae* genomes were found to differ in the internal regions of lower copy number in three chromosomes. There regions comprise in chromosome I, *PRM9*, *MST28*, *YAR047C*, *YAR050W*, *CUP1*, *YAR060W* and *YAR061W*; chromosome VII, *YGL052W* and *MST27*; chromosome XII, *ASP3* and *YLR156W*. *PRM9*, *MST27* and *MST28* genes encode nonessential membrane proteins specific to *Saccharomyces* sensu stricto species. *YAR050W* encodes a lectin-like protein that participates in flocculation; Asp3p is a nitrogen catabolite-regulated cell wall L-asparaginase II. *CUP1* had two times lower number of copies than the average for *S. cerevisiae* species. This was believed to

cause increased sensitivity to copper in *S. boulardii* when compared to other *S. cerevisiae* strains. There were also two regions on chromosomes III (*LEU2*) and V (*URA3*) with a high number of copies due to the fact that these genes are absent in *S. cerevisiae* BY4743 (reference strain). Lower number of copies was seen in several genes of DUP240 multigene family.[21]

Within genes with higher copy number, two functions are well represented: protein synthesis (*RPL31A*, *RPL41A*, *RPS24B*, *RPL2B* and *RSA3*) and stress response (*HSP26*, *SSA3*, *SED1*, *HSP42*, *HSP78* and *PBS2*). It is possible that these genes aid in increased growth rate and pseudohyphal switching and in higher resistance to high pH.[21]

Study III showed that many genes had variable copy number. There were 50 genes without reads mapped onto them and 94 genes with read coverage lower than 20 reads. From these 144 genes, 85 were dubious ORFs, 32 uncharacterized genes and 27 known genes. The 27 genes can be seen in Table 3. All of these genes were located in the telomeric or subtelomeric regions except for ASP3. On the other hand, other genes were found to have higher copies numbers in *S. boulardii* Biocodex and unique28 (Table 4). Duplicated and triplicated genes mostly encoded stress response proteins, elongation factors, ribosomal proteins, kinases, transporters and fluoride export, which might aid in *S. boulardii*'s adaptation to stress conditions.[16]

Table 3: Genes that were absent in S. boulardii Biocodex and unique28 in study III. [16]

Systematic	Genes	Gene function
YOL165C	AAD15	Aryl-Alcohol Dehydrogenase
YNR074C	AIF1	Mitochondrial cell death effector
YHL047C	ARN2	Transporter
YLR155C	ASP3-1	Cell-wall L-asparaginase II involved in asparagines catabolism
YLR157C	ASP3-2	Cell-wall L-asparaginase II involved in asparagines catabolism
YLR158C	ASP3-3	Cell-wall L-asparaginase II involved in asparagines catabolism
YLR160C	ASP3-4	Cell-wall L-asparaginase II involved in asparagines catabolism
YLL063C	AYT1	Acetyltransferase
YOL164W	BDS1	Bacterially-derived sulfatase
YLR465C	BSC3	Bypass of Stop Codon
YNR075W	COS10	Protein of unknown function
YGR295C	COS6	Protein of unknown function
YOL158C	ENB1	Endosomal ferric enterobactin transporter
YOL156W	HXT11	Putative hexose transporter that is nearly identical to Hxt9p
YJL219W	HXT9	Putative hexose transporter that is nearly identical to Hxt11p
YOL157C	IMA2	Isomaltase
YIL172C	IMA3	Isomaltase
YJL221C	IMA4	Isomaltase
YGR289C	MAL11	High-affinity maltose transporter (alpha-glucoside transporter)
YGR288W	MAL13	MAL-activator protein
YIR041W	PAU15	Seripauperin
YKL224C	PAU16	Seripauperin
YJL217W	REE1	Cytoplasmic protein involved in the regulation of enolase (ENO1)
YAL064C-A	TDA8	Topoisomerase I Damage Affected
YOR068C	VAM10	Vacuolar Morphogenesis
YIL173W	VTH1	Putative membrane gycloprotein
YJL222W	VTH2	Putative membrane gycloprotein

Table 4: Genes with varying copies in S. boulardii Biocodex, Unique28 and S. cerevisiae S288C in study III. [16]

Genes in multiple copies	*Sb* biocodex	*Sb* unique28	*Sc* S288C
Seripauperin PAU	18	20	7
Gag-pol fusion proteins	16	16	49
Thi13	5	6	1
IMP dehydrogenase IMD3	4	2	1
Cos3p	4	5	1
YIL169C-like protein	3	4	2
Aad4p	3	2	1
Fex1p	3	3	1
Ribosomal 60S subunit protein L2B	3	4	1
Hsp32p	3	3	1
Y' element ATP-dependent helicase protein 1 copy 1	2	5	5

In *S. boulardii* absent genes identified in study I encode mostly Ty elements (about 60 ORFs). As for the remaining absent genes, 25 are of unknown function and 15 had been previously characterized in *S. cerevisiae*. While in this study Ty1/2 were found to be absent in *S. boulardii*, study II found that all Ty1, 3 and 4 elements, but not Ty2 and Ty5, were absent in all *S. boulardii* strains. [16]

Since Ty elements are transcribed under diploid control and not in MATα/MATa cells, mating-type status of *S. boulardii* was analyzed in study I. This yeast was shown to be diploid with both mating types, but unable to sporulate and become haploid. Since Ty elements renewal through transposition probably occurs during sporulation and mitosis of haploid form, *S. boulardii* cannot replenish these elements due to its inability to sporulate and enter haploid form. Hence, *S. boulardii* probably ends up losing its intact Ty elements because of recombination between the long terminal repeats of individual elements.[15] Study II found that *Saccharomyces* normally has MATa and α sequences in chromosome III. Homothallic switching endonuclease (HO) is a site-specific endonuclease that initiates mating type interconversion. This enzyme cleaves the mat locus on chromosome III and the consequent double-strand break leads replacement mat locus information with the opposite one. HO is therefore crucial foe gene conversion in the MAT locus during haploid form and is expressed by homothallic cells. On the other hand, heterothallic strains (T189A, G223S, L405S and H475L) have 36 amino acids substituted or eliminates, which results in loss of HO activity. All strain of *S. boulardii* were homothallic and diploid, with both MATα and a present in its genome.[16]

It has been shown that *S. boulardii* has better pseudohyphal switching during nitrogen starvation than other *Saccharomyces* strains. Several genes related to pseudohyphal growth (*CDC24*, *CDC42*, *DFG16*, *RGS2*, *CYR1*, *CDC25*, *STE11*, *SKM1* and *RAS1*) had considerably different number of copies in study III. *CDC42*, *DFG16*, *RGS2*, *CYR1* and *CDC25* had a higher number of copies, while *STE11*, *SKM1* and *RAS1* had a lower number of copies than those of their chromosomes. Cdc24p is a guanine nucleotide exchange factor for Cdc42p; Cdc42p is a small rho-like GTPase; Dfg16p is a membrane protein that participates in pseudohyphal growth); Rgs2p is a negative regulator of cAMP signaling; Cyr1p is an adenylate cyclase and Cdc25p is a guanine nucleotide exchange factor for Ras2p and Ras1p. Ste11p is a MEK kinase that participates in pheromone and pseudohyphal and invasive growth mitogen-activated protein kinase signal transduction pathways; Skm1p belongs to the PAK family of serine/threonine protein kinases and Ras1p is a GTPase that participates in the cAMP pathway. This suggests that cAMP pathway is probably altered and its hyperactivation leads to increased pseudohyphal growth. *S. boulardii* ability to create pseudohyphae was analized and compared with that of *S. cerevisiae* FY1679 and *S. cerevisiae* _1278b (filamentation positive). It was observed that *S. boulardii* was filamentous with a faster and more extensive response than *S. cerevisiae*_1278b.[21]

Two flocculation genes were found to display increased copy numbers in *S. boulardii* and *S. cerevisiae* YJM1385 /fruit borne) and YJM1129 (brewery strain), while other *S. cerevisiae* strains have only one copy. *FLO1* has seven copies in *S. boulardii* and *S. cerevisiae* YJM1385 (fruit borne) and YJM1129 (brewery strain), while other *S. cerevisiae* strains have only one copy. *FLO8* has similar number of copies in *S. boulardii* and most strains of *S. cerevisiae* except for *S. cerevisiae* S288C,

BY4741, BY4742, FY1679, JK9-3d, SEY6210, W303, X2180-1A and YPH499. The last (laboratory strains) had truncated proteins due to point mutations, which makes them unable to flocculate properly and adhere to foreign surfaces. The higher or lower number of copies of some flocculation genes in *S. boulardii* can affect its adherence and flocculation ability, as well as sensitivity to stress.[16]

Many studies have shown that *S. boulardii* is unable to use galactose as a carbon source. Galactose metabolism, more specifically conversion of galactose to glucose-6-phosphate is part of the Leloir pathway and involves many: enzymes galactose-mutarotase, galactokinase (*GAL1*), galactose-1-phosphate uridyltransferase (*GAL7*), UDP-galactose-4-epimerase (*GAL10*) and phosphoglucomutase (*PGM1* and *PGM2*). Interestingly, it was reported that *S. boulardii* has all galactose uptake and fermentation genes. However, *S. boulardii* EDRL is able to assimilate, but not ferment galactose, possibly due to energy requirements. Not only does *S. boulardii* induce the enzymatic activities of lactase-phlorizin hydrolase, α-glucosidases, alkaline phosphatases and aminopeptidases, but also increases D-glucose intestinal absorption, one of the products of lactose degradation. Production of lactase by the host, partially stimulated by *S. boulardii* leads to lactose degradation, which can help in lactose intolerance. Genes involved are *MIG1*, *PGM1*, *GAL7*, *GAL10*, *GAL1*, *CYC8*, *GAL2*, *GAL4*, *GAL80*, *PGM2*, *GAL3* and *TUP1*. [16,19]

Palatinose uptake and metabolism involves the enzyme isomaltase (encoded by *IMA1*, *IMA2*, *IMA3*, *IMA4*, and *IMA5*), with affinity for palatinose. Although *S. boulardii* is unable to use palatinose, it possesses the genes *IMA1* and *IMA5*, but not *IMA2*, *IMA3* and *IMA4*, encoding isomaltase[16], and it is also able to stimulate the expression of these digestive enzymes in intestinal epithelial cells.

Factors that affect probiotics' survival in the host

Probiotics must be able to endure in adverse conditions. The main obstacles in the stomach are the very acidic pH (2 to 3) and the presence of proteases like pepsin that kill most microorganisms, including probiotics that enter the organism with food. Diseases like hypochlorhydria, in which the patient produces low quantities of acid in the stomach, decrease the bactericide properties of the stomach and make the patient more susceptible to infections by Helicobacter pylori and *Salmonella spp* and to migrations of potentially pathogenic microorganisms to the small intestine where they establish themselves. In the case of the small intestine, the principal problems are the high concentrations of bile salts, pancreatic enzymes, hydrolytic enzymes, pancreatin, organic acids, the integrity of the epithelial and brush border, the immune defense and the native microbiota and its secondary metabolism products (H_2S, bacteriocins, organic acids, among others).[11] Bile salts are toxic to some microorganism because they affect their cellular membranes' lipid bilayer structure.[11] However, many probiotics are able to resist degradation by hydrolytic enzymes and bile salts. [22,7] Other parameters should also be considered in order to best mimic the gastrointestinal system: the corporal temperature, the peristaltic movements and transport, the length of stay in the intestinal traffic and the absorption of small water molecules.[8]

It is important to point out that different probiotics used in therapeutic procedures have different susceptibilities to acidic pH and to bile acids stress. There are several supplements that contain *S. boulardii*, like, for example, Enterol ("Biocodex") and Probiz ("Unique Biotech Limited").

Enterol consists of capsules with 250 mg of lyophilized *S. boulardii*, while Probiz is made of capsules with *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Bifidobacterium bifidum* and *S. boulardii*. A study has shown that these two supplements, in among others, are able to survive conditions that simulate the passage through the stomach and intestine *in vitro*. The used gastric-like conditions consisted of a solution of sodium chloride (0,72 g/L), potassium chloride (0.05 g/L), sodium bicarbonate (0.37 g/L) and pepsin (3.0 g/L) with the pH adjusted to 2.5. On the other hand, the conditions used to simulate the intestinal environment consisted on a solution of bile salts (3.0% wt/vol) and pancreatin (0.1%) with the pH adjusted to 7.0. Both simulations were made separately and one right after the other. The medium used for Enterol was Sabouraud (Dextrose) Agar (SDA) which is used for yeast and mold. However, Probiz contains several microorganisms and different media had to be used to assess the different probiotics: SDA was used for *S. boulardii*, De Man, Rogosa and Sharpe agar **(**MRS) agar for *Lactobacillus* and *Bifidobacterium*. Both supplements had high quantities of viable cells initially, yet, after incubation with gastric juice, *Lactobacillus* e *Bifidobacterium* did not survive in any of the supplements. Enterol showed complete resistance after incubation of gastric juice, with *S. boulardii* growth on SDAat the same level as before the exposure. Probiz contained only one species with high level of survival to gastric juice: *S. boulardii*. After exposure to both conditions, only *S. boulardii* survived in the 3 media. In all supplements, besides the 2 referred, only *Bacillus coagullans* e *S. boulardii* showed stability in gastric juice and bile acids, while *Bacillus clausii* was partially resistant. In mixed supplements, only these species survived.[7]

Another study showed that *S. boulardii* grows faster than *S. cerevisiae* at both 30 and 37 °C, which is important in probiotic strains, since the human's body core temperature can go from 35.8 to 38.2 °C (medium of 37°C). *S. boulardii* is also more resistant to very high temperatures keeping 65% viability after one hour at 52ºC, while *S. cerevisiae* loses viability down to 45%.[11]

When grown in gastric simulated environment (pepsin, sodium chloride and a pH of 2), *S. boulardii* is more resistant than *S. cerevisiae*. In an intestinal simulated environment (pancreatin, pepsin, sodium chloride and a pH of 8) both strains viability was not affected. [11] It was also shown that *S. boulardii* is more resistant to low pH than all *S. cerevisiae* strains tested in study III, particularly at pH 2.0.[21]

Tolerance displayed by *S. boulardii* to bile salts has also been tested, as bile salts are detergents produced in the liver from cholesterol and secreted to the intestine to improve nutrient absorption. As detergent like molecules, bile salts can alter the membrane lipid bilayer of microbes residing in the gastrointestinal tract, becoming eventually toxic to them. Surprisingly, *S. cerevisiae* is more tolerant to bile salts than *S. boulardii*. However, since resistance to bile salts is considered for organisms that can support at least 0.3% (w/v), none of these strains can be considered resistant. This is interesting since most yeasts can survive up to 0.75% (w/v).[11]

Many studies show that *S. boulardii* is quickly removed from the gastrointestinal system in healthy individuals, suggesting that this yeast does not strongly adhere to intestinal epithelial cells. Indeed, both *S. boulardii* and other *Saccharomyces* strains are unable to remain attached to human and mouse epithelial cells *in vitro* and *in vivo*, respectively. They are also unable to directly adhere to

polarized colinic cell line cells, a model that includes interaction with common and pathogenic microorganisms. However, they do adhere to Caco2 cells through an extracellular factor, probably secreted mucus. After 48 hours of administration, none of the yeast were still found in feces. After 72 hours of a single administration, viable cells had low levels both in flushing and in mucosal scrapings, which suggests that these yeasts do not colonize the gut. The stomach, jejunum, ileum, secum and colon contents of sacrificed mice at one and three hours and their feces after single administration was analyzed through cultures of each. After one hour, most viable cells were located in cecum and colon. At three hours, viable cells were almost absent in stomach and small intestine and started being flushed in the feces. No strain had a considerable better or worse survival in feces or flushing and/or transit time than others. Significant differences in mice were observed, probably due to variations in laboratorial techniques (such as volumes administered and proportion of fecal pellets obtained), mice physiology, feeding habits, among others. This shows that high resistance *S. boulardii* to very low pH does not result in a higher number of viable cells reach the intestine didn't affect levels in the intestine, which were similar for all strains. However, it has also been shown that *S. boulardii* is able to colonize the intestine of gnotobiotic mice after single administration. In the case, viable cells were present in the feces for more than ten days after administration. It was also shown that repeated administration to healthy mice was essential for colonization over several days. In humans the results were similar: *S. boulardii* quickly removed after single administration but remained for about three days with daily administration. Moreover, administration of ampicillin increased fecal concentration too. This means that although *S. boulardii* can colonize the intestine, competition with intestinal microbiome limits it unless the microbiome is depleted, such as under antibiotic therapy.[21]

Another study used an *in vitro* dynamic model representing the human gastrointestinal system to test the survival of *S. boulardii*. For the stomach and lower intestine, the TIM-1 model was used, while for the superior intestine the artificial colon model (ARCOL) was used. TIM-1 is a dynamic and multi-compartmental model controlled by a computer. This model is divided into four successive compartments where, in each, different conditions are simulated: stomach, duodenum, jejunum and ileum. This system tries to represent as precisely as possible the conditions of each site: pH, body temperature, peristaltic movements and transport, gastric, bile and pancreatic secretions and absorption of small molecules and water. Each compartment is made of a glass unit with an inner flexible membrane that allows the reproduction of peristaltic movements when water is pumped in between the glass and membrane at regular intervals. In the TIM-1 model, *S. boulardii* showed a high resistance to gastric and lower intestinal conditions independently of the mode of administration (while fasting and with a cup of water or with an occidental type meal) In the ARCOL model, *S. boulardii* was not able to colonize the colon, but had an effect in the microbiotic profile that was dependent on the individual.

The study of physical barriers that protect the probiotics from adverse conditions has been gaining more and more interest. *S. boulardii* survival rate within gastrointestinal conditions was tested when it is free or encapsulated in a hydrogel double layer with sodium alginate and gelatin as a first and second covering agents, respectively. Since the first layer is frequently porous, probiotics can

pass through it, hence the second layer is used to hinder the passage through the layers. The strain was inoculated in acidic medium (pH=2) or in a solution that simulates the intestinal fluids (bile salts 3%w/v) for 120 minutes. With the first medium, it was still possible to detect the presence of the free yeast in the medium for both free and immobilized yeast, nonetheless resistance to pH was higher when the probiotic was immobilized inside the beads. In the intestinal medium, *S. boulardii* population decreased 1.5 logs after exposure for 120 minutes when free, while immobilized *S. boulardii* concentration remained constant. It was concluded that encapsulation increases significantly the survival rate of *S. boulardii* to low pH and in simulated intestinal conditions, especially if encapsulated by a double layer. [23]

However, all these studies only analyze the resistance to bile salts in a suspension for a few hours. When grown in solid YPD medium supplemented with different concentrations of bile salts for 48h, *S. boulardii* is very susceptible to bile salts, even if in low concentrations. *S. cerevisiae* follows the same behavior, although being slightly less susceptible than *S. boulardii*. In order to be defined as bile salt resistant, a microorganism has to endure very high concentrations of bile salts from 3 to 9 g/L. However *S. cerevisiae* and *S. boulardii* can only endure a maximum of 1.5 and 1 g/L of bile salts, respectively, and hence cannot be considered bile salts resistant.[11]

Safety issues on *S. boulardii*'s use

Certain safety issues arise with the use of probiotics. One of these is the translocation of live organisms from the intestine to other areas of the body. Animal studies show that there is reduced translocation in the treatment with this yeast when compared with other strains of *S. cerevisiae*. The persistency of the probiotic in the intestine could also be a complication, yet *S. boulardii* does not persevere in the intestine after three to five days after discontinuation of the ingestion, according to pharmacokinetic studies. These three problems have therefore a minimal impact for this strain. [4]

Yet another predicament could be adverse reactions. Be that as it may, this strain is used in Europe since the 1950s and has been researched in clinical trials all over the world, having showed an exceptional safety profile. In 90 controlled and random trials, none of them reported any serious adverse reactions, while only some presented moderate adverse reactions. One of these trials observed that the only adverse reactions were thirst and constipation in patients with Clostridium difficile. Although systemic infection in the host and fungemia are a potential problem, there were no fungemia cases reported in clinical trials, however some cases (24) were observed in case reports or case series in the literature. A major part of these cases happened in adults with serious co-morbidity and central venous catheters which responded well to fluconazole or amphotericin B. Other cases happened when the infant was premature or there was a parallel chronic disease, immunodeficiency and/or debilitation. Moreover, there are no reports of sepsis in healthy patients, only in patients with preexisting intestinal diseases.[4] In some cases fungemia may be due to contamination of central lines due to *S. boulardii* becoming airborne when its capsules are open.[5] Other variants of *S. cerevisiae* (non *boulardii*) have also been reported to cause fungemia, but their prognostic is worse than that caused by *S. boulardii*. It should be noted though that it is a challenge to determine if fungemia is

caused by *S. cerevisiae* or *S. boulardii* in older reports, since at the time there were no methods that allowed distinguishing between the two strains. [4]

S. boulardii's clinical efficacy

Gut dysbiosis refers to the microbiome's quantitative and qualitative composition alterations, which can lead to changes in the host-microbiome interactions. These changes may contribute to a disease state frequently associated to inflammation that is normally non transmissible. Probiotics are a promising treatment or adjuvant to diseases that lead to the variation of the microbiome. Even though most of these diseases are gastrointestinal, they can also occur in other areas, for example, the oral cavity, skin, liver and vagina. [2,4]

From 90 controlled and randomized trials (CRT), 88 studied this strain and tested 15 different diseases (by decreasing number of clinical trials): pediatric diarrhea (27% of the trials), antibiotic-associated diarrhea (AAD) (22% of the trials), *Helicobacter pylori* infection (16% of the trials), inflammatory bowel diseases, irritable colon syndrome, acute diarrhea in adults, traveler's diarrhea, necrotizing enterocolitis, enteral tube feeding diarrhea, *Clostridium difficile* infections, HIV-associated diarrhea, giardiasis, sepsis, acne and hepatic diseases. [1]

Acute pediatric diarrhea can have several causes, from infections (for example, caused by rotavirus, *E. coli*, *Shigella*, among others) to alterations in lifestyle and nutrition. This disease can lead to serious dehydration in children which can result in hospitalization, sepsis and death. *S. boulardii* efficacy in the treatment of this disease is measured through various methods: number of children cured until a specific day, improvement of diarrhea symptoms or reduction of the average diarrhea length (days). 24 CRT studied the treatment of this disease in children between 3 months and 18 years with oral rehydration therapy. *S. boulardii* was administered for about one week and 83% of trial reported a significant increase in treatment efficacy without serious adverse reactions. Through meta-analysis of the data from the several trials, it was found that this strain is able to diminish the duration of the diarrhea by one day, which is relevant when considering young and undernourished children. [1,24]

Antibiotic-associated diarrhea depends on several factors: type of antibiotic, host related factors (age, health state, among others), etiology, hospitalization state and presence of a nosocomial outbreak. This disease occurs more frequently during health care associated outbreaks (hospitals, third age nursing homes, among others), where susceptible patients are subjected to antibiotics and infectious agents. In trials for this disease, the probiotic is administered while also taking the antibiotic or until a certain additional period after stopping the antibiotic medication and before the diarrhea has appeared. This way, it is possible to observe if the patients develop this disease or not until 4 to 8 additional weeks after stopping the administration of antibiotics, since AAD can appear latter instead of during the treatment. From 21 CRT with *S. boulardii* used for prevention of this disease, it was found through metanalysis that this strain has a significant efficacy in the prevention of ADD, which does not happen with other strains of *S. cerevisiae*. [1,25,26]

H. pylori is a bacteria that colonizes the gastric mucosa and normally only results in an asymptomatic and chronic state. However, some people develop gastric and/or duodenal ulcers. Besides that, the presence of this microorganism is also a risk factor to the development of gastric lymphomas or adenocarcinomas in elderly patients. About 50% to 80% of the world population carries this bacteria and treatment consist on a triple or quadruple therapy (antibiotics and a proton bomb inhibitor), for two weeks. This treatment results in a high number of secondary effects, especially on the gastrointestinal tract. For this reason, some patients prematurely stop the treatment before it finishes and eradicates *H. pylori*. Clinical trials were done with patients following this treatment with supplementation of probiotics, in order to test if the latter increases the bacteria eradication rate, prevents adverse reactions or diarrhea associated with the treatment. In 10 trials with *S. boulardii*, this strain was effective in the elimination of this pathogen in 82% of the patients, reduced the risk of adverse reaction in 58% and the incidence of diarrhea in 53%. Thus we can see that this probiotic has a high interest when used as adjuvant of standard treatment for this disease. [1,27]

Inflammatory bowel disease encompasses several immunity associated inflammatory diarrheal diseases like ulcerative colitis, pouchitis and Crohn's disease. In Crohn's disease, the most affected areas are the end of the small intestine and of the colon resulting in symptoms like diarrhea, abdominal pain, colic and lack of appetite. As opposed to ulcerative colitis, lesions created by this disease are profound and irregular, giving rise to thickened areas that may cause intestinal obstructions and endanger the patient's life. Since the intestinal wall is disturbed, microorganisms are able to translocate the epithelial layer and stimulate the immune system. Most often, patients are subjected to frequent surgeries throughout their lives. This disease does not have a treatment, meaning that only the symptoms are alleviated. However, 10 to 60% of patients have a relapse after the symptom's treatment ends. Furthermore, the study of this disease in clinical trials is difficult, since the disease is sporadic, without defined etiology, requiring long treatments and follow-ups. CRT with probiotics for this disease focus on the improvement of symptoms and remission of the disease. In 3 trials with *S. boulardii*, 2 have shown that patients had less relapses, however on the third trial (of a larger scale) there was no significant difference between administering or not *S. boulardii* as an adjuvant. Hence, more CRT should be done to confirm these results. [1,28,29,30]

Irritable bowel syndrome is a disease frequently characterized by several symptoms like abdominal bloating, abdominal pain and disturbed intestinal transit, which decrease life quality and increase health cost. Standard treatments only focus on relieving the symptoms and show no better results when compared with a placebo. From 4 trials with *S. boulardii*, 50% found a significant improvement of the patient's symptoms with decreased defecation' frequency and improved life quality.[1,31–34]

Acute diarrhea in adults can develop quickly, but is normally of short duration and sporadic. The causes may be unknown or due to infectious agents like *Entamoeba histolytiva*, *E. coli* or *Salmonella*. The former diarrheal diseases mentioned are not included in this classification. In 4 trials, *S. boulardii* was effective in 75% of the cases, having decreased the diarrhea's severity or eliminating it. However, the 4 trials were of different etiologies, which limit the conclusions to be taken. [1,35,36]

Traveler's diarrhea may have an enterotoxigenic or/and enteroaggregative origin due to *E. coli*, *Campylobacter jejuni*, *S. typhimirum*, vírus (Norwalk, rotavírus, among others) or parasites (*E. histolytica*, *Giardia lamblia*, among others). From 3 trials, 2 analyzed *S. boulardii*'s efficacy in preventing this disease, while the last studied patients that already suffered from it. In the first case, it was found that the strain diminishes the disease's incidence in a dose dependent way, whereas in the second case there were no significant differences found in terms of the diarrhea's duration. Although the number of trials is limited, this indicates that the strain may be more effective in the prevention of the disease than in its treatment. [1,37,38]

Enteral nutrition-related diarrhea is a complication commonly associated with enteral tube feeding, which can result in deficient nutrition of a previously ill patient. This disease happens in about 50 to 60% of the patients fed this way and can lead to severe complications such as acidosis, morbidity increase, health care's cost increase and death. In 3 trials, all of them reported that *S. boulardii* leads to fewer patients developing diarrhea and those that do develop it, display a shorter duration of the episode. Regardless, more studies should be done to confirm these results. [1,39,40]

Necrotizing enterocolitis is the most common gastrointestinal disease in low weight newborns with an incidence of 9% and a mortality rate of 20%. Only one study in three showed promising results for the use of *S. boulardii*. However, this strain only significantly decreased the development of sepsis, but didn't prevent the disease from happening. [1,41–43]

Clostridium difficile in the leading cause of hospital acquired gastrointestinal infection leading to higher hospitalization periods (from 4 to 36 days), mortality increase, higher health care's cost and higher need for surgical colectomies. This disease normally appears in hospitalized adults, however cases of non-hospitalized children and adults have been increasing. There are only three standard antibiotic treatments and the efficacy of one of them has been decreasing. Furthermore, 20 to 60% of patients may develop recurring episodes of the infection even after additional antibiotic treatment. Three controlled, randomized and double-blinded trials have shown that *S. boulardii* is significantly effective in decreasing re-occurrence episodes. One of the trials was done simultaneously with the antibiotic treatment and reported two adverse reactions: thirst and constipation. Another trial studied the treatment of different antibiotics with different doses of probiotic used as adjuvant. Only one of the antibiotics, allied with high concentrations of the strain, showed complete eradication of the infection. This was also the only trial in which *S. boulardii* proved to be effective in decreasing the number of relapses. This may indicate that the strain may be more effective in decreasing re-occurrence episodes only if there is complete elimination of the infection and its toxins before treatment with the probiotic. However, more studies should be done to corroborate these findings. [1,44–46]

Giardiasis is a disease characterized by moderate to severe diarrhea of long duration. Its symptoms are weight loss, abdominal pain and weakness. Only two trials were performed with *S. boulardii* and both show a significant effect: in the first trial, there was a decrease of *Giardia*'s cysts after the diarrhea ended due to an antibiotic treatment, while, in the other, there was a significant increase of cured children in relation to chronic diarrhea. [1,36,47]

HIV-associated diarrhea is very frequent and can become a potentially fatal chronic issue. Although several small non-controlled trials obtained promising results in using *S. boulardii*, only one of two CRT reported the strain's efficacy in significantly increasing the number of people who stopped having diarrhea. [1,48,49]

Mechanism of action

The colonization of the gastrointestinal system in babies is most probably determinant in the establishment of the intestinal microbiome later in life. The formation of this microbiome starts before delivery and continues throughout childhood. The initial colonization can be affected by several factors: genetic constitution of the newborn, the delivery method (caesarean or vaginal delivery), antibiotic's use, the feeding mode (breast-feeding or formulas), progenitor's stress level and the presence of inflammatory conditions in the progenitor. Before delivery, bacteria present in the placenta, umbilical cord and meconium may affect the colonization, whereas, in the newborn, bacteria present in the progenitor's vagina and breast-feeding milk are preponderant for the colonization. However, these microorganisms do not necessarily remain in the gastrointestinal tract and can disperse to extra-digestive areas through dendritic cells or macrophages. These are able to penetrate the epithelium and transport the microorganisms from the intestinal lumen to other areas by entering the immune cell circulation through the blood circulation. [1,4]

The normal human has about 40000 bacterial species in its intestinal microbiome. This flora has many functions, including facilitating digestion, resisting colonization by pathogens, among others. The latter implicates interaction of several microflora's bacteria and results in a barrier against the colonization by pathogens by competing for nutrients and adhesion sites and by production of bacteriocins and enzymes that inhibit the growth of pathogens. [1,4]

The mechanism by which homeostasis in the intestine is maintained is not yet fully understood. One theory is based on the epithelial hypoxia state that limits available oxygen in the colon. This state leads to the maintenance of an equilibrated microbiome which produces metabolites that contribute to nutrition, immune training and security of the host's intestinal niche. When there are alterations in the microbiome, probiotics may help restore the microbial diversity and change the disturbance to the microbiome by action mechanisms that are not fully elucidated. However, the effect of administration of probiotics is in part known: increased production of SCFAs, humidification of fecal matter, increase in the defecation frequency and increase of feces volume. [4]

Probiotics are believed to display a variety of mechanisms: antitoxin effects, physiological protection, modulation of the normal microbiome, metabolic regulation and signaling pathway modification, nutritional and trophic effects, immune system regulation, pathogen's inhibition, interactions with the brain-gut axis, cellular adhesion, cellular antagonism and mucin production. [1,4]

Modulation of the normal microbiome

Modulation of the normal microbiome may be favored directly by transiting probiotics which produce antimicrobial substances (such as reuterin or plantaricins) or even indirectly by modulation of the immune system, epithelial receptors and intestinal barrier.[9] There are several factors that can

damage the barrier offered by the microbiome to colonization, such as antibiotic's use and surgeries. Both may result in host susceptibility to colonization by pathogens until the microbiome is reestablished. This may take 6 to 8 weeks after treatment with antibiotics or until the disease is cured. The use of probiotics as modulators of the normal microbiome, through its colonization and normalization during the susceptibility period, may work as a substitute of the normal microflora until it is reestablished, *S. boulardii* seems to help restoring normal microflora in this type of patients. [1,4]

Pathogen's inhibition

For a probiotic, in general, pathogen's inhibition and/or reduction involves a modulation of several metabolic and signaling pathways, although the specific pathways involved in this process are not fully known. It was proposed that this mechanism may be exerted through inducing a pH decrease in the intestinal lumen, interfering with the pathogen's adherence to the intestinal lumen, competing for nutrients sources and producing bacteriocins or similar substances. Besides that, several components of the probiotic metabolome (such as organic acids, bacteriocins, hydrogen peroxide, diacetyl, amines, among others) interact with multiple targets in metabolic pathways that regulate cellular proliferation, differentiation and apoptosis; inflammation and angiogenesis and metastization.[5] *S. boulardii* is capable of interfering directly or indirectly with intestinal pathogens and possibly of inhibiting directly the growth of some of them, such as *Candida albicans*, *S. typhimurum*, *Yersinia enterocolitium* and *Aeromonas hemolysin*. [9]

Physiological protection

The mucosal barrier in the intestine is composed by the mucus layer, the epithelial lining of mucosal tissues and immune cells. It is important that all these are well modulated to maintain the barrier's fortitude in order to avoid gastrointestinal diseases. Tight junctions envelop each superficial cell and create a mechanical barrier that hampers crossing of microorganism through the intestinal barrier. They are formed by transmembrane proteins (occluding, tricellulin, claudins, junctional adhesion molecules and peripheral membrane proteins, such as Zo1p, Zo2p, Zo3p and cingulin). On the other hand, fluid with mucus and IgA secretions sequestrates microorganisms. During gastrointestinal diseases, there can be disruption of the mucosal barrier probably due to gene expression's regulatory mechanisms, tight junction structure and cytoskeletal signaling alterations. However, mucosal injury is mainly due to disproportion of pro (IL-1, IL-2, IL-6, IL-8, TNF-α and INF-γ) and anti-inflammatory (IL-4 and IL-10) cytokines. Secretion of these cytokines is controlled by NF-κB and mitogen activated protein kinases (MAPK) pathways. Not only can these cytokines cause mucosal injury, but they can also alter tight junction proteins' genes expression and, hence, epithelial permeability. For example, interferon-γ (IFN-γ) can change claudin-2 and occludin's gene expression and cause decreased barrier integrity, while TNF-α is able to lower tight junctions' strands numbers, resulting in higher transepithelial ion permeability. Studies show that some probiotics can not only directly interact with intestinal epithelial cells and help strengthen the mucosal barrier, but also produce soluble factors that avoid epithelial cell apoptosis. Studies *in vitro* show that the latter may be due to activation of anti-apoptotic protein kinase B (Akt) and suppression of nuclear factor κB (NF-κB). All these may contribute to pathogen exclusion and homeostasis maintenance. It was also observed that *S. boulardii* preserves the tight junctions' structure between enterocytes in the intestine, which

reduces fluid loss due to diarrhea. This preservation is due to inhibition of pro-inflammatory cytokins (IL-8, IL-1β, IL-6, and TNF-α), increase of peroxisome proliferator activated receptor-γ (PPAR-γ) levels in the colon and avoidance of MAP kinases Erk1/2, NF-κB and c-Jun N-terminal kinase (JNK)/SAPK activation. NF-κB activation inhibition is due to repression of IκB-α's phosphorylation and degradation, while MAPK activation inhibition was a result of ERK1/2 phosphorylation repression. These kinases and molecules are involved in inflammation signaling pathways used, for example, by *Salmonella typhimurium* to increase inflammation. This species sometimes binds to *S. boulardii* and becomes less capable of translocation and, hence, of activating inflammatory signaling pathways. At the same time, *S. boulardii* is also able to transiently increase IgA concentration in the intestinal fluid. Similarly, a study shows that this strain decreases the intestinal permeability in patients with Crohn's disease. This shows that *S. boulardii* is capable of using anti-inflammatory mechanisms to lower pro-inflammatory cytokines levels and, hence, preserve tight junctions. *S. boulardii* is also able to inhibit myosin light chain phosphorylation and transmonolayer electrical resistance decrease, which are correlated to tight junction permeability.[1,50–54]

Cellular adhesion, cellular antagonism and mucin production

In order for the host not to mechanically eliminate the microbiome's microorganisms, it is crucial that they can adhere to the host's surfaces. [1,4] Mucin is produced by epithelial cells to avert adhesion by pathogenic bacteria, hence probiotics should be able to adhere to the intestinal mucous even in this situation. However, while some probiotics may become a part of the microflora, other simply go through the intestine and modulate or influence the existing microbiome before exiting the body. *S. boulardii* is capable of producing around 44 cell wall and/or adhesion proteins (encoded by *AGA2*, *BGL2*, *CCW12*, *CIS3*, *CKA2*, *CRH1*, *CRR1*, *CWH41*, *CWP2*, *DCW1*, *DFG5*, *DSE2*, *EXG1*, *EXG2*, *FIG2*, *FIT1*, *FIT2*, *FKS3*, *GSC2*, *HKR1*, *KNH1*, *KRE6*, *KTR1*, *LAS21*, *MNT2*, *PIR3*, *PST1*, *ROT2*, *SCW10*, *SCW11*, *SCW4*, *SHE10*, *SKN1*, *SMK1*, *SPI1*, *SPR1*, *SRL1*, *SUN4*, *UTR2*, *YPS1* and *YPS3*), by which they mediate the adhesion of pathogenic bacteria to yeast cells. This interaction limits the ability of the pathogen to bind directly to the intestinal receptors and proceed with host invasion. Furthermore, since *S. boulardii* is unable to bind to epithelial cells of healthy individuals and is quickly flushed out, when pathogens bind to *S. boulardii* it is possible that they are flushed together with the yeast cells. A study analyzed adherence of 11 enteropathogenic bacteria to *S. boulardii* and found that only *Escherichia coli*, *Salmonella Typhimurium* and *Salmonella Typhi* were able to adhere to *S. boulardii*. [9,16,55]

Generally, adherence may be due to specific or non-specific binding. The later relies on electrostatic and hydrophobic interactions, which have lower affinity than specific binding. In the case of intestinal epithelium, adherence is done through mannose molecules, which are abundant in yeast cell walls. Interestingly, *E. coli* binds to *S. boulardii* more strongly than to *S. cerevisiae*. The affinity between the two is due to *S. boulardii*'s cell wall mannan oligosaccharides. Interestingly, yeast cells do not need to be viable for adherence to occur, which makes the use of non-viable probiotic in immunocompromised patients an interesting possibility. It should be noted that bile salts have been shown to decrease adherence of bacteria to intestine epithelia, however no such studies were made for yeast adhesion. This decrease may be explained by alteration of sugar component due to bile salt

action that leads to changes in surface and adhesion properties: diminished surface hydrophobicity and higher surface potential. Among *S. boulardii* mutants devoid of each of the above mentioned 44 cell wall or adhesion proteins, only five lost adhesion properties: Δ*cis3*, Δ*cwp2*, Δ*fks3*, Δ*pir3* and Δ*scw4*. Cis3p is a glycoprotein containing mannose present in the cell wall and a member of the PIR family. Cwp2p is a covalently linked cell wall mannoprotein; and a major constituent of the cell wall, which plays a role in cell-wall stabilization and acid resistance. Fks3p is a protein involved in spore wall assembly similar to 1,3-beta-D-glucan synthase catalytic subunits Fks1p and Gsc2p. Pir3p is a glycosylated covalently bound cell-wall protein required for cell-wall stability. Scw4p is a cell wall protein with similarity to glucanases. [32]

Ingestion of contaminated food is the main origin of *Salmonella enterica serovar typhimurium* (ST) infections. It has been shown that ST adheres to *S. boulardii*. This leads to entrapment of the pathogen in the gut lumen, without it being able to adhere to intestinal epithelial cells or translocate to other tissues. The two microorganisms aggregate and are then flushed faster than the bacteria alone. In the very early stages of infection, *S. boulardii* induces pro-inflammatory cytokine production, more specifically IFN-γ, and represses the production of anti-inflammatory cytokines, more specifically IL-10 in the small intestine. IFN-γ boosts macrophage activity, while IL-10 represses it. This suggests that *S. boulardii* function as an immune-modulator by initiating phagocytes recruitment and stimulating the host innate immune response. In the cecum there is induction of other pro-inflammatory cytokines in early stages of infection, however, afterwards, IL-10 levels were increased, which may indicate a return to normal immune response levels.[56]

S. boulardii also produces flocculins and agglutinins (encoded by *FLO1*, *FLO5*, *FLO8*, *FLO9*, *FLO10*, *FLO11*, *FIG2* and *AGA1*/*SAG1*) principally during stationary phase. Flocculins are attached to the yeast's cell wall and bind selectively to mannose in other yeast's cell wall.[9,32]

Some probiotics possess several surface adhesins that help in the attachment to the mucous layer by, for example, recognizing several host molecules' classes such as transmembrane proteins (integrins or cadherins) and extracellular matrix components (collagen, fibronectin, laminin or elastin). [1,4] They can also promote mucous adhesion by themselves (for example, by producing bacterial adhesions, such as mucus-binding protein, MUB) or with the help of saccharide moieties and lipoteichoic acid. It has been shown that *S. boulardii* is capable of adhering to intestinal mucus membrane and avoiding adhesion of other pathogens flowing by to the intestine. Since probiotics compete with pathogens for reception sites in the intestinal tract (competitive exclusion), the adherence of *S. boulardii* to the mucus membrane leaves fewer biding sites open for the pathogen, thus they go through the intestine and exit the body sooner. [15] It has also been demonstrated that, due to *S. boulardii* bigger size when compared with bacteria, this yeast can hamper biofilm formation of other pathogenic strains by steric hindrance. However, this contradicts previous studies mentioning *S. boulardii* inability to strongly adhere to the intestinal epithelia of healthy individuals and ability to bind to pathogens and being flushed out together. [15]

However, probiotics can also influence the production of mucin and the barrier function of the intestine through the production of butyrate by the host, a SCFA that is able to regulate and increase

mucin and defensins gene's expression (*MUC2* and *MUC3*), which hinders the invasion and adherence of pathogenic microorganisms.[57]

Antitoxin effects

Antitoxin effects refer to interference with pathogen's toxins in the intestinal lumen. It may be due to different mechanisms: suppression of toxin production, decrease of intestinal pH, attenuation of virulence, modification of toxin receptors and stimulation of the nonspecific immune system (as in immune cell proliferation, increase macrophage's phagocytic activity and boost secretory IgA's production). *S. boulardii* blocks the receptor or functions as a decoy receptor for the pathogen's toxin.[16,19]

S. boulardii produces a 54 kDa serine protease, which gradually degrades (by hydrolysis) directly toxin A and B originated from *C. difficile* and the cellular enterocytic surface's receptor to which the toxins bind. The last process decreases toxin A and B ability to bind to the brush border membrane, however it seems to be less active than toxin degradation, maybe due to protection of receptors by a mucus coat and unstirred water layer that block proteases diffusion. Toxin A is a cyto and enterotoxin that increases inflammation, fluid secretion, and mucosal permeability and injury in the intestines. On the other hand, toxin B is a cytotoxin that increases inflammatory cytokines release from monocytes. Even though toxin A is the major root of mucosal injury and inflammation in mammals, toxin B is also able to cause mucosal injury in humans. Toxin A enteroxytic effects are dependent on binding to brush border membrane receptors. The response of the host immune system to these toxins, especially release of antibodies, determines their toxicity. *S. boulardii* has been shown to decrease fluid secretion and mucosal permeability and damage. The mentioned phosphatase is released mostly in the end of the small bowel and colon and results in diminished toxin concentration in flushing, but no change in bacteria amount, which is consistent with toxin degradation by a protease. It should be noted that, although *S. boulardii* decreases enterotoxin effects, it does not decrease fibroblast cell rounding. One possible reason is that fibroblast rounding might only require toxin A fragments, while enterotoxicity might require intact toxin A. It is also possible that there is still enough non degraded toxin A to cause fibroblast rounding, but not enteroxicity. This yeast also specifically increases IgA anti-toxin A secretion as a response to *C. difficile* infection and not as a general probiotic mechanism. It has also been shown that it represses activation of ERK1/2 MAPK signaling pathway and, hence, pro-inflammatory cytokines (such as IL-8) secretion induced by toxin A. *S. boulardii* can, hence, be used as a mucosal adjuvant to stimulate the host immune system. Other mechanisms that *S. boulardii* uses against *C. difficile* infection are growth inhibition and decreased toxin production due to secreted factors and stimulation of host mucosal disaccharidase activity. Four 54 kDa serine proteases were found in *S. boulardii* genome and belonged to carboxypeptidase and subtilisin-like sub-classes (eventually encoded by *PCR1*, *RRT12*, *YSP3* or *YBR139W*). It is worth noting that other strains of *S. cerevisiae* do not produce this enzyme, although they appear to harbor in their genomes all the genes that may encode it.[16–19]

Another study refers that *S. boulardii* produces a 63 kDa alkaline phosphatase (eventually encoded by *PHO8*, *PRP3*, *JIP4*, *YDR476C*, *SNF1*, *SNM1*, *PEX29*, *DIG2*, *CWC21*, *KRE2*, *VPS52*,

VPS72, *VP60*, *RIB3* or *PAC11*), which inhibits *E coli*'s endotoxins (such as LPS) by dephosphorylating their activation sites and diminishes its toxicity. This phosphatase is active in a large scale of pH (2 to 10). In this case, *S. boulardii* also significantly decreases TNF-α levels. However this molecule is also present in most *S. cerevisiae* strain with a conserved activation site. [16,18]

cAMP is a secondary messenger involved in several signaling pathways. For example, *Vibrio cholerae* relies on vasoactive intestinal polypeptides and prostaglandins to recognize receptors in adenylate cyclase in order to activate a cAMP-dependent signaling pathway and, hence, promote chloride secretion. It also increases cAMP levels and secretion for this purpose and produces cholera toxin (CT) with subunit A and B. *S. boulardii* is capable of interfering in this pathway and affecting consequent chloride secretion. More specifically, *S. boulardii* produces a 120 kDa protein that has been shown to decrease water and sodium secretion in intestinal loops and counteract the increase in cAMP levels in rat intestinal cells done by *Vibrio cholerae*'s toxin, leading to this strains toxicity inhibition. Fifteen 120 kDa proteins were found in *S. boulardii* genome, belonging to kinase and transporter families (endcoded by *KIN1*, *MAD1*, *TFC4*, *VAS1*, *KAP120*, *PIK1*, *NMD5*, *JSN1*, *PUF2*, *RGC1*, *ENA5*, *KCS1*, *SEG2*, *NUP120* or *MSH3*). *S. boulardii* is also able to adhere to CT's subunit B, which results in internalization of subunit A. CT's subunit A is required for activation of cAMP and trehalose, but after adherence to *S. boulardii* and internalization is no longer available for this purposes. It was proposed that this adherence is due presence of a receptor identical to enterocyte receptors (ganglioside receptor Gm1p) both structurally and functionally.[1,16,18,20]

Bacillus anthracis can cause intestinal anthrax when this microorganism is ingested through contaminated food. The bacteria leads to ulcerative lesions from the jejunum to cecum. It produces a three component toxin, whose components are: protective-antigen (PA), lethal factor (LF) and edema factor (EF). PA binds to host cell receptors and associate with LF or with EF. LF is a metalloprotease dependent on zinc that specifically cleaves MAPK kinases and Nlrp1bp. This results in inhibition of MAPK kinases and, hence, cytotoxic effects such as increased cytoskeleton remodeling. EF is a adenylate cyclase dependent on calmodulin able to increase cAMP levels. The combination of PA and LF creates a lethal toxin (LT), while of PA and EF creates an edema toxin (ET). LT disrupts intestinal epithelium integrity, causing mucosal erosion, ulceration and bleeding. It also changes barrier function through tight junction and adherent junction complexes. These two are transmembrane proteins that bind adjacent cells through actin cytoskeleton. Reorganization of actin cytoskeleton leads to thick and parallel actin stress fibers formation and stabilization, which leads to endothelium stiffness, blebbing and barrier disruption. Furthermore, LT induces Mek2p cleavage. *S. boulardii* helps maintain barrier function, maintain Zo1p perijunctional distribution, lower number of cells with stress fibers and reduce Mek2p cleavage. However, for the last effect it is necessary that *S. boulardii* is pre-incubated in order to release proteases into the medium. *S. boulardii* can also cleave PA or adhere to it, which makes PA unavailable for binding to LF or EF and translocation. Hence, LT and ET cannot be formed and toxin effects are reduced. Cleavage of PA is done by proteases implicated in protein maturation. Kex2p and 54 kDa serine protease (from C. difficile' toxins A and B studies) may be responsible for this cleavage.

Adherence of PA to yeast cells does not depend on growth stage, but does depend on yeast cell number. [20]

Trophic effects

Trophic effects include secreted digestive enzymes by the probiotic, increased brush border digestive enzymes and nutrient transporters activity. *S. boulardii* is the source of or, at least, a modulator of enzymatic activities required to maintain a healthy gastrointestinal tract. Several studies have shown many trophic effects in *S. boulardii* in these two categories: increase in brush border sucrase, lactase, and maltase activities[58–62,63]; increased isomaltase activity[62]; increased glucoamylase and N-aminopeptidase total activity[60]; increased leucine-aminopeptidase activity[64]; increased α,α-trehalase activities in the endoluminal fluid and intestinal mucosa; increase in brush border α-glucosidase[63]; increase in spermine, spermidine in rat's jejunal mucosa[59,61]; increased adenosine triphosphatase, γ-glutamyl transpeptidase, lipase, and trypsin activities and increased tumor necrosis factor α, interleukin 10, transforming growth factor β, and secretory IgA[5]; increased GRB2, SHC, CrkII, Ras, GAP, Raf and ERK1,2 signaling molecules in rats and decreased p38 MAPK and NF- Kb[65]; increased diamine oxidase activities, putrescine, brush border sodium/glucose cotransporter expression and sodium-dependent d-glucose uptake.[61,66,67]

Production of lactase by the host and its overexpression by *S. boulardii* leads to lactose degradation, which can help in lactose intolerance. Genes involved are *MIG1*, *PGM1*, *GAL7*, *GAL10*, *GAL1*, *CYC8*, *GAL2*, *GAL4*, *GAL80*, *PGM2*, *GAL3* and *TUP1*.

Many of these digestive enzymes (sucrase-isomaltase, maltase-glucoamylase, lactase-phlorizin hydrolase, alanine aminopeptidase and alkaline phosphatase) and nutrient transporters (sodium-glucose transport proteins) activity may be induced by polyamines secreted by *S. boulardii*. *S. boulardii* secretes polyamines (in the dependence of the activity of the enzymes encoded by *SPE2*, *SPE3*, *CAR1*, *CAR2*, *PUT2*, *PUT1*, *PRO1*, *PRO2* and *PRO3*) that promote RNA binding and stabilization and, hence, growth and differentiation proteins (lactase, maltase, sucrase, among others) synthesis. These molecules are also able to defend lipids from oxidation and boost SCFA activity. The enzymes will probably then participate in the GRB2-SHC-CrkII-Ras-GAP-Raf-ERK1,2 pathway and PI3K pathway. Polyamines may also affect kinase activities and external signals, furthering modulation these two pathways. They can also aid in creation of specific transcripts by interacting with DNA. All of these polyamines functions lead to a general polyamine-triggered metabolic activation in order to regenerate brush border damaged areas quickly. [1,4,66,67]

In terms of modulation, it can restrain aminotransferase activity in the liver of non-alcoholic adults; increase disaccharidases and sucrases expression; increase naftol-AS-BI-phosphohydrolase and leucin arylamide activity and decrease of β-glucuronidase activity. *S. boulardii* is also able to boost oligopeptides hydrolysis, which allows aminopeptidase to move in the lumen and, hence, increase permeability and possibly repressed the production of food antigens.

Metabolic activity regulation and signaling pathway modulation

Probiotics can modulate short chain fatty acids (SCFA: acetate, propionate and butyrate, between others) and/or branched-chain fatty acid (BCFA: isobutyrate, 2-methylbutyrate, isovalerate, among others) synthesis. In particularly, the use of probiotics to regulate SCFA levels has been gaining more and more importance. SCFA have a complex role in the human organism and they affect it physiologically and biochemically in different tissues (intestine, liver, adipose, muscle and brain tissues). They are also a major source of energy for enterocytes and essential signaling molecules in the regulation of energy homeostasis and metabolism. This may be due to the fact that they can enter systemic circulation and interact with cell receptors in peripheral tissues. They have very diverse roles and may be responsible for the improvement of many diseases, for example, by: enhancing satiety in obese patients due to changes in neuronal excitability; decreasing fat accumulation in adipose tissue resulting in decrease lipolysis and inflammation and increased adipogenesis and leptin release; improving the carbohydrate metabolism, fasting blood glucose levels, insulin sensitivity, antioxidant status and metabolic stress in patients with type 2 diabetes; lowering the concentration of certain biomarkers for cardiovascular disease in patients with insulin resistance syndrome; disrupting the outer membrane of gram-negative bacteria, leading to inhibition of its growth; helping in water and electrolytes absorption in the colon; among others. Normally these molecules are produced by fermentation of undigested food in the intestine. [1,4]

S. boulardii produces SCFAs, such as acetic acid and propionic acid, just acetic acid in itself makes up 50% of total SCFAs in the colon. The major energy source in intestinal epithelial cells is butyrate and, hence, it affects proliferation, differentiation, mucus secretion and barrier function. It can also decrease bacterial translocation, enhance tight junctions structure, boost mucin production, repress NF-κB activation and has antioxidant and anti-inflammatory properties. A study reported that a shot-term treatment (6 days) with *S. boulardii* diminishes the incidence of diarrhea and increases the fecal and total SCFA concentrations in patients suffering from *C. difficile* infections until about 9 days after the treatment is stopped. This increase in concentration, especially in butyrate's, may be the reason why *S. boulardii* is an effective prevention treatment of total enteral nutrition-based diarrhea too. [1,4]

A study analyzed the antimicrobial activity of 12 *S. boulardii* and 11 *S. cerevisiae* strains through an agar-well diffusion assay with indicator strain *E. coli* MG1655. Only two *S. boulardii* strains showed a clear inhibition zone. The supernatant of the cell-free culture of these two strains was analyzed and it was found that acetic acid (6 g/L) was the cause of the antimicrobial properties. Addition of acetic acid at the same concentration and at a 4.2 pH also showed an antibacterial effect on *E. coli*.[68]

It was observed that all *S. boulardii* strains produced acetic acid, but to different extents, whereas *S. cerevisiae* strain produced lower quantities. Production increased during the first 24 to 36 hours of growth. At 24 hours, most *S. boulardii* strains reached 2.9 g/L of acetic acid, but the two antimicrobial strains continued to accumulate acetic acid until 48 hours of growth and produced about 5.20 g/L of acetic acid and maintained this concentration until 72 hours of growth, accompanied by a

decrease of pH from 6 to 4.2. On the other hand, other *S. boulardii* strains eventually started to consume acetic acid, which diminished its accumulation and turned it transient. However, when there is high glucose levels, all *S. boulardii* strains produce very high amounts of acetic acid. Although all *S. boulardii* strains were able to consume acetic acid at 30ºC, the two *S. boulardii* antimicrobial strains were unable to consume the produced acetic acid at 37ºC. This means that the accumulation of acetic acid at high concentration and, hence, the higher antimicrobial properties of the two strains is dependent on temperature and manifest itself at the human body temperature. The decrease in pH due to acetic acid concentration is essential for the antimicrobial activity of short-chain organic acids (higher number of protonated and uncharged form when at pH lower than the pKa). In this form, the molecule is able to diffuse into cells causing intracellular acidification, which compromises metabolic activity and proliferation of pathogens. The combined effected of high acetic acid concentration and lower pH can explain *S. boulardii* effectiveness as a probiotic. Not only acetic acid leads to antimicrobial properties, but acetate also stimulates the expansion and build-up of T regulatory cells, facilitates goblet cell proliferation, induces mucus secretion gene expression inhibits proinflammatory cytokine CXCL8 and serves as a substrate for the production of butyrate by the microbiome. [68]

The gastrointestinal tract has an oxygen gradient from the proximal to the distal area and a radial gradient with high oxygen concentration near the epithelial surface and very low oxygen concentration in the center of the gut lumen. So, since acetic acid only seems to be produced under aerobic conditions by *S. boulardii*, its production should be higher near the epithelial surface. Also, during antibiotic treatment and pathogen infection, oxygen concentration increases in the gastrointestinal tract, which makes *S. boulardii* specially potent in antimicrobial properties in these conditions. [68]

The two copies of sdh1^{H202Y,F317Y} allele in all *S. boulardii* strains is crucial for the higher acetic acid production when compared with *S. cerevisiae*. On the other hand, the presence of one or two copies of the whi2^{S287*} allele makes the difference between transient and moderate or continuous and high acetic acid production, respectively, at 37ºC. Sdh1p is a flavoprotein subunit of the succinate dehydrogenase complex that is involved in the TCA cycle and mitochondrial respiratory chain. Deletion of *SDH1* leads to an increase in acetic acid production in *S. cerevisiae*. It is possible that sdh1^{H202Y,F317Y} allele may cause malfunctioning of the succinate dehydrogenase complex at 37°C. This malfunctioning might result in reduced initiation of the TCA cycle due to shortage of oxaloacetic acid, which leads to accumulation of acetyl- CoA and hence of acetic acid or in compromised TCA cycle, which leads to accumulation of pyruvate and, hence, of acetaldehyde and then of acetic acid. Whi2p has phosphatase activator activity and is able to form complexes with plasma membrane phosphatase (Psr1p). This protein participates in several processes such as cell cycle regulation, cell proliferation, general stress response, endocytosis, actin cytoskeleton organization and amino acid sensing. Full activation of STRE-mediated gene expression for degradation of unproperly folded proteins required Whi2p. The incapability to consume acetic acid at 37ºC by the two *S. boulardii* strains is not due to deficient acetic acid assimilation, but probably due to decreased acetic acid tolerance. This is corroborated by the fact that whi2^{S287*} allele is similar to a *WHI2* deletion mutant and this mutant has

reduced acetic acid tolerance, which leads to an indirect inhibition of acetic acid consumption and possibly to early growth arrest. The later might result in redistribution of carbon to acetic acid production instead of biomass formation. [68]

Both sdh1^{H202Y,F317Y} allele and whi2^{S287*} allele had a causative SNP each that were only present in *S. boulardii* and not in *S. cerevisiae* strains. This means that these two specific point mutations are, at least partially, responsible for the antimicrobial properties of *S. boulardii*. [68]

Immune system regulation in the intestinal lumen:

Certain molecules produced by the microbiome can perform immunomodulatory and anti-inflammatory functions that stimulate immune cells. This ability arises from the interaction between the probiotics and the epithelial cells, dendritic cells monocytes, macrophages and/or lymphocytes. [1,9]

It is possible that *S. boulardii* regulates immune responses by acting as an immune stimulant or by reducing the pro-inflammatory responses. *S. boulardii* may cause an increase in the IgA secretor levels in the intestine; an increase in the IgG levels in serum when *C. difficile*' toxins A and B are present; binding and modulation of dendritic cells through TLRs that leads to increased IgA, IgM and cytokines secretion (IL-1β, IL-12, IL-6, TNF-α and IL-10)[9]; an interference in signal mediated NF-κB transduction pathways which stimulate the production of pro-inflammatory cytokines; a blockage of ERK1/2 and MAP kinases' activation which would normally promote the production of IL-8 and cellular necrosis in mouse models with ileal loop and in *in vitro* models and the imprisonment of T helper cells in mesenteric lymphatic nodes, reducing inflammation. It should be noted that IgA boosts the intestinal mucosa immunologic barrier. [1,9]

In conclusion, probiotics may be able to persistently modulate both the innate and adaptive immune responses either locally or systemically. [1,9]

Mechanisms of adaptive response to host induced stresses

In order to survive in the gastrointestinal tract, yeasts have to endure very acidic pH (2 to 3), presence of proteases like pepsin, high concentrations of bile salts, presence of pancreatic enzymes, hydrolytic enzymes, pancreatin and organic acids, the immune defense and the native microbiota and its secondary metabolism products (H_2S, bacteriocins, organic acids, among others), the corporal temperature, the peristaltic movements and transport.[8,11] Hence, it is important to know how yeasts respond to stress in general or to specific stresses like heat, oxidative, osmotic, salt stress, among others. [69–71,72,70,73,74]

A general and coordinated transcriptional response was found to be common to most stresses and denominated the General Stress Response or the Environmental Stress response. This response consists in a huge, fast, transient and genome-wide gene expression change. Although genes that suffer expression changes are the same across most stresses, no two stresses have an identical response. On the contrary they have different initiation timing, amplitude and regulation of these expression changes, which depend on the specific stress. This implies that a general response is initiated and precisely controlled according to each stress. Consistently, exposure to a light specific stress condition was found to lead to higher resistance to later stress conditions, of the same kind or

not. This cross-resistance to different stress conditions may mean that the general stress response answers in proportion to the difference between crucial physiological systems and a homeostatic set-point. [69–71,72,70,73,74]

Although most genes induced during the general stress response are regulated by the transcription factors Msn2p and Msn4p, these do not modulate single-handedly all these genes in all cases. Also, not all genes in a given pathway must suffer expression alteration and, in many cases, only rate-limiting steps or regulatory molecules are affected. This means that yeast tries to target specific cellular processes with as few changes to gene expression as possible in order to decrease energy requirements and quicken eventual return to normal growth conditions. [69–71,72,70,73,74]

Transient increase in gene expression is probably used to help in transition to new stress conditions, while genes whose expression remains altered probably have a continuous role in the new stress conditions. It should be noted that, although transcript levels are increased, this does not mean that the transcript products are active. In other words, transcript levels are increased as preparation for potentially needed activity, making the yeast ready to quickly modulate the activity of synthesized proteins at the post-translational level. After some time, gene expression is fully adapted and maintained at new transcript levels, which are closer to levels before stress conditions and even more similar between stress conditions than upon exposure to sudden stress. [69–71,72,70,73,74]

Among the most commonly up-regulated functional categories are those related to carbohydrate metabolism (including glycerol, glycogen and trehalose metabolism), metabolite transport, maintenance of the cellular redox potential, detoxification of reactive oxygen species, autophagy and vacuolar functions, protein folding and degradation, cell wall modification, mitochondrial functions and intracellular signaling. On the other side, ribosome proteins, RNA metabolism, translation and amino acid and ergosterol synthesis are commonly down-regulated [69–71,72,74–78]

During stress conditions, yeast must be able to regulate its energy metabolism accordingly, which leads to several gene expression changes in carbohydrate metabolism. Glucose transporters, that import extracellular glucose into the cell, are up-regulated and glucokinases activate glucose for its degradation and proceed with trehalose and glycogen synthesis and storage, glycolysis for ATP synthesis and pentose phosphate shuttle (*ZWF1*, *GND2*, *SOL4* and *NQM1*) for NADPH regeneration. Glycolysis and gluconeogenesis genes are usually up-regulated under stress, especially those related to fructose-2,6-biphosphate synthesis and degradation (*FBP26*, *PFK26*, *YLR345W*). Detoxification of this pathway bypath is also represented (*GRE2* and *GRE3*), as well as *HXK1*, *HXK2*, *GLK1*, *PGM2* and *EMI2* that encode enzymes required for several glycolytic steps and the *HXT2*, *HXT5*, *HXT6*, *HXT7*, *HXT15*, *HXT16*, *HXT17*, *MTH1*, *MAL11*, *GPM2*, *EMI2* and *STL1* genes related to sugar transportation. [69–71,72, 74] Processes involved in respiration are also up-regulated, including TCA cycle rate limiting steps (*CIT1*), cytochrome c isoform (*CYC7*), and factors that affect cytochrome c oxidase (*COX15*) and ubiquinone (*COQ5*) components synthesis and assembly. This allows a higher use of respiration components, which increases ATP synthesis. [69–71,72,74]

The most relevant storage compounds in yeast are glycogen and trehalose and their synthesis is altered when exposed to environmental stress. Glycogen is essential for cell survival in stress conditions. During stress conditions, genes involved in its synthesis (*UGP1*, *GLG1*, *GLG2*, *GSY1*, *GSY2* and *GLC3*) and degradation (*GPH1* and *GDB1*) have been shown to be induced under stress. Other genes related to glycogen, but not directly involved in its synthesis are also up-regulated, such as *UGP1*, which directs the substrate for glycogen synthase; *GAC1* and *PHP21*, which encode initiators of glycogen synthesis; *GLC7*, which modulates glycogen synthase activity; and *PIG2*, which interacts with *GSY2* and *GLC7*. [69–71,72,74]

Trehalose can work as a compatible solute. In environmental stress, trehalose accumulates and it has been shown that this helps protect yeast against dehydration by stabilizing cellular membranes and against denaturation and unfolded protein aggregation by stabilizing protein structures, avoiding heat inactivation and denaturation in *S. cerevisiae* and *S. boulardii*. Under stress, yeast accumulates trehalose and consequently the genes required for its synthesis (*PGM2*, *UGP1*, *TPS1*, *TPS2* and *TSL1*) are induced. However, genes involved in its degradation (*NTH1* and *ATH1*) are frequently induced too. During moderate stress, trehalose concentration does not seem to be associated with glycerol production. Hence trehalose probably does not serve as a reserve compound for glycerol synthesis during osmotic stress. [69–71,79,72,80,73,74]

Glycerol is a by-product of the glycolytic metabolism, serving as a redox valve. In yeast, glycerol synthesis is a crucial stress response. Not only the genes related to glycerol synthesis, but also the genes related to plasma membrane sugar transporters and to glucose phosphorylation may be affected.[69–71,75,72,81] Besides trehalose and glycerol, acetate synthesis is also increased under stress. [69–71,79,72]

Cellular structures (such as proteins, lipids and DNA) can be damaged by chain of oxidation reactions when reactive oxygen species accumulate. This process disturbs the internal redox potential, which has consequences in enzymatic activity. In most stresses, yeast tries to maintain their internal redox potential by employing defense against oxidative stress like reducing oxidizing compounds or compounds that can cause oxidative damage (glutathione, thioredoxin, among others) and by using certain enzymatic activities (superoxide dismutase, catalase, among others). Glutathione, thioredoxin and protein sulfhydryl groups can be oxidized or reduced and the balance between the two forms modulates the intracellular reducing conditions. This regulation requires NADPH. However, genes involved in oxidative stress response aren't exclusively induced during this stress, but in most stress. Normally isoenzymes of thioredoxin and glutaredoxin (*TRX2* and *TTR1*), γ-glutamyl transferase (major glutathione-degrading enzyme) (*ECM38*) and thiol specific antioxidants (*PRX1* and *TSA1*) are up-regulated. *TTR2*, *TSA2*, *GTT1*, *NCE103*, *MAG1*, *MMS2*, *GRX1* and *DDR48* genes are also usually up-regulated. As it has already been said, pentose phosphate shuttle (*ZWF1*, *GND2*, *SOL4* and *NQM1*) is also up-regulated for NADPH regeneration, which may help restore NADPH reducing equivalents. Another method of defense against oxidative stress consists in detoxifying reactive oxygen species. In this case, genes encoding cytosolic superoxide dismutase (*SOD1*), catalase (*CTT1*), glutamate decarboxylase (*GAD1*) and glutathione peroxidases (*HYR1* and *GPX1*) are usually up-regulated.

Since the mitochondria is the major origin of intracellular reactive oxygen species creation, this organelle possesses its own defense mechanism for oxidative damage. This included cytochrome b5 reductase (*MCR1*) and cytochrome c peroxidase (*CCP1*), among others (*COX5B*, *SCO2*, *ETR1* and *CYT1*). [69–71,79,72,80,73,74]

Several intracellular signaling genes are also usually induced. In protein kinase A positive and negative regulators (PKA), two catalytic subunits (*TPK1* and *TPK2*) and cAMP dependent inhibitory subunit (*BCY1*), a phosphodiesterase (*PDE1*) and a kinase (*YAK1*) are concomitantly induced. The induction of the two subunits is unexpected, since their pathway induce Msn2p and Msn4p cytoplasmic location and hence repress stress response. [69–71,79,72,80,73,74]

Ribosome transcripts, ribosomal proteins and tRNA transcripts are responsible for a significant part of RNA polymerase I, III use and compose more than 95% of total RNA. During most stresses ribosome proteins, rRNA synthesis and processing, ribosome biogenesis, tRNA synthesis and processing and some genes encoding RNA polymerase I and III are usually repressed. Repression of ribosomal protein genes is usually modulated by transcription factor Rap1p. Translation and, hence, protein synthesis is also normally repressed, including translation initiation, elongation and termination factors. [69–71,72] All of these shows that there is a transient halt in protein synthesis during stress conditions. Normally protein synthesis is inhibited transiently for most proteins but maintained or induced for some specific proteins. Also, although translation repression and restoration happen in response to all stresses, response dynamics differ. After the adaptation period, translation goes back to normal levels and is no longer inhibited. Repression of these energy consuming processes saves energy that can be relocated to synthesis of molecular chaperones and other mechanism involved in stress response. [69–71,72,70,73,74]

Although many protein folding chaperones are specifically induced by heat-stress due to the presence of heat denatured proteins, a subset of these chaperones (heat shock proteins *HSP12*, *HSP26*, *HSP42*, *HSP48* and *HSP70* family: *SSA4*, *SSA3*, *SSA1*, *SSE2*, *HSP78* and *HSP104*) is also induced under most stress conditions. Degradation is necessary for proteins that cannot be refolded or that are damaged or denatured in order to avoid their aggregation and genes involved in this process were up-regulated. For this purpose, cytoplasmic material (such as proteins, small molecules and organelles) is enveloped by vesicles (autophagy) and later degraded in vacuoles for further recycling. Hence genes related to autophagy and its regulation (*APG1*, *APG7*, *AUT7* and *AUT1*) are usually up-regulated. Vacuolar proteins (*PMC1*, *VAB2*, *LAP4*, *PEP4*, *PRB1*, *PCR1*, *YPS6*, *PAI3* and *PBI2*) are also usually up-regulated. Ubiquitination genes *HUL4*, *UBC5*, *UBC8*, *CUE1*, *UBI4*, *UBP15* and *ATG7*), related to targeting of proteins for turnover, are usually up-regulated. Proteasome genes (*SCL1*, *PRC1*, *RPN5*, *YPS6*, *PBI2* and *PAI3*) are also up-regulated. All of these processes may accelerate the cells ability to change its protein internal repertory in response to stress conditions. However, these processes involve significant energy consumption through ATP hydrolysis, leading to an increase in (or/and relocation of) energy production. [73,81]

Amino acid synthesis genes are usually down-regulated, while catabolism genes are up-regulated. Methionine synthesis genes are especially repressed. This makes sense, since, as it was

seen, protein synthesis is mostly repressed, while degradation of unfolded proteins is up-regulated. All of this may explain why the yeast suffers a temporary growth arrest. [69–71, 75,72,81]

Oxidative stress response is modulated by cytoplasmic transcription factor Yap1p that accumulates in the nucleus during oxidative stress. Many genes whose expression is altered during oxidative stress contain ARE's-API-responsive elements in their promoters that are recognized by Yap1p. Another transcription factor, Skn7p, regulates the expression of genes that overlap with Yap1p modulation. Skn7p not only is controlled by transcription factor Sln1p that senses osmotic stress, but it also can interact with the heat shock transcription factor Hsf1p and has a DNA binding domain similar to that of Hsf1p. Also, Skn7p can interact with several other transcription factors such as Swi4p/Swi6p and Crz1p. Swi4p/Swi6p participates in cell cycle dependent gene expression, while Crz1p modulates calcineurin-dependent calcium-induced responses. It is hence possible that Skn7p integrates disparate signals when it comes to control gene expression.

Heat shock causes partial or total denaturation of proteins (and other molecules) and their consequent aggregation or separation of complexes. It also causes increased cell membrane fluidity. In response to heat stress, yeast responds with the so-called heat shock response (HSR) very rapidly (within minutes), which is controlled by the heat shock transcription factor Hsf1p, and change its physiology (membrane composition, carbohydrate flux, among others). In HSR there is induced transcription of heat shock protein's genes, a family of about 12 proteins that are evolutionarily conserved (*HSP100*, *HSP90*, *HSP70*, *HSP60*, *HSP104*, *HSP40* and small HSPs). A good part of these proteins is able to function as molecular chaperones that are essential in regulation of protein function and structure: they help in protein folding and conformation maintenance, in order to maintain their structural integrity. The genes that are exclusive to heat shock and not in other stresses present induced expression that remains increased and is not transient. Msn2p and Msn4p also modulate heat shock gene expression. In fact, most of HSR is regulated by Hsf1p, Msn2p and Msn4p. Hsf1p is a heat shock transcriptional activator that is inactive in normal conditions. It is possible that Hsp90p chaperone binds to Hfs1p in order to inactive it. Msn2p and Msn4p are activator involved in most stress responses that induce the expression of several stress genes. This means that HSR may be a subset of the general stress response that is however especially activated during heat shock. [73,82,74]

Hyperosmotic stress results in changes that are among the quickest and most transient. On the other hand, there are few genes whose expression is specifically altered. However, many genes with induced gene expression in response to general stress are more strongly induced in hyperosmotic stress. [74,75]

During salt stress, the high osmolarity glycerol mitogen activated protein kinase pathway (HOG MAPK) senses the cellular envelope's turgor pressure through transmembrane proteins Sho1p and Sln1p and induces the activation of Hog1p and other MAPK phosphorelays by phosphorylation. They later change the carbohydrate metabolism and osmotic processes through signal transduction and regulation of several transcription factors activity (such as *MSN2*/*MSN4*, *MSN1*, *HOT1* and *SKO1*). For example, *HOG1* can bind to the DNA and activates expression of *CTT1*, a well-known stress-response gene, and transcription factors *MSN1*, *MSN2* and *MSN4*. Hog1p pathway involves *SLN1*,

SHO1, *SSK2*, *SSK22* and *PBS2* genes and is activated early in saline stress and then gives way to other pathways. One of these pathways is modulated by Msn2p/Msn4p transcription factors that bind to stress response elements (STREs) and amplify the signal. Both *MSN2* and *MSN4*, normally located in the cytoplasm, can enter the nucleus during stress response and interact with *HOG1*. However, these two transcription factors probably can bind to other consensus sites upstream of the genes or bind concurrently with stress specific factors. This regulation of the protein synthesis is crucial and affects proteins globally.

Salt stress results in expulsion of water and accumulation of excessive quantities of sodium, monovalent cations and metabolites, which affects the osmotic potential, generates water deficit and leads to a drop of internal pressure, decreased cellular activity and metabolic toxicity. It also impairs membrane potential, affecting membrane transport activity and ion homeostasis, which disrupts the intracellular pH equilibrium. Even though salt stress response includes and is very similar to osmotic stress response, there are still differences: salt stress has additional responses in ion homeostasis. As a response to osmotic stress, the yeast tries to remodel plasma membrane integrity and fluidity in order to create turgor pressure against the plasma membrane that neutralizes water passage against the osmotic gradient into the cell. [69–71,79,72,73]

Genes related to ion homeostasis (*ENA1*, *VMA6*, *VPH1*, *VMA7*, *VMA5*, and *VMA1*/TFP1*)* are normally transcriptionally induced. Ena1p is a crucial ATPase for sodium, lithium and potassium ion efflux (as it was seen in ergosterol synthesis), which is induced during salt stress. The remaining genes encode different components of H^+-ATPase complex, which is essential for the creation of proton electrochemical gradient in sodium sequestration into the vacuole. [69–71,72]

Other factors involved in stress response regulation can be found in *Table 5*, including the percentage of general response genes they affect and the conditions in which they operate. [69–71,72,74]

Table 5: Factors involved in stress response genes expression regulation. [69–71,72,74]

Factor		**Percentage of general response genes affected**	**Conditions in which it is operated**
Type	**Factor**		
Multifunctional proteins	Rap1p	23% repressed and 5% induced	
Silencing	Tup1p-Ssn6p	10% repressed and 4% induced	
Chromatin remodeling	Rpd3 mutant	28% repressed	
	Rpd3p/Isw2p	23% induced	
Transcription factors	Msn2p/Msn4p	88% induced	Most stresses
	Yap1p	6% induced	Oxidative stress
	Hot1p	3% induced	Osmotic stress
	Hsf1p	Unknown	Heat stress
	Msn1p	Unknown	
	Sko1p repressor	Unknown	Osmotic stress
Protein kinase pathway: MEC pathway	Ste11p/Ssk1p pathways	100%	Osmotic stress
	PKC pathway	Likely 100%	Heat stress, secretion defects
PKA pathway	TOR pathway	Likely 100%	Nutrient repletion
	SNF1 pathway	Unknown	Glucose starvation
	PHO85 pathway	Unknown	Glucose repletion

A study showed that *S. boulardii* suffers plasmolysis during salt stress. This yeast was shown to be haloduric, since it can survive at high salt concentration, but is unable to grow.[79]

Cellular membranes are the primary sensor and protector of environmental stress. The membrane's composition and its regulation is very important for stress resistance and depends on its permeability and fluidity. In *S. cerevisiae*, the membrane's structure is formed by distinct lateral microdomains (lipid rafts) which are composed by associations of sterols and sphingolipids with proteins.[83] Actually, the membrane is composed of lipids and proteins in about a 1:1 proportion and this microdomains host several relevant protein that participate in Na^+, K^+ and pH homeostasis, nutrient and enzyme transport, mating, signal transduction, cytoskeleton anchoring, drug efflux, stress response, adhesion molecules and antigen activation. Among these functions, the most relevant are the presence of a barrier to solute diffusion associated with formation of solute gradients and transmembrane ions for energy storage, and the presence of specific binding sites that induce catabolic signaling pathways.[84] Membrane lipids include phospholipids or glycerophospholipids, sterols and sphingolipids. [85,86]

Glycerol-3-phosphate and fatty acid are necessary for phospholipids synthesis. Sphingolipids are involved in signal transduction across the plasma membrane. When suppressed, yeast are unable to grow in stress conditions such as low pH, high temperatures, high salt concentrations, possibly due to impairment of proton extrusion through ATPases impairment or increase of permeability. [85,86]

Sterols have an important role as signaling molecules and as components of cell membranes. They bind to the membrane's phospholipids and are able to modify the membrane fluidity and permeability and to regulate the activity of membrane-bound enzymes. All of these help in stabilizing the membrane's structure. Regulation of the membrane's rigidity affects membrane proteins lateral movement and activity. Sterols are also involved in substance transportation through the regulation of the activity of membrane H^+-ATPases and other membrane transporters, in vesicle formation, activity of membrane transporters, protein sorting, cytoskeleton organization, endocytosis and mating.[84] Normally it is protected by sphingolipids present within the membrane.[87, 85,86]

Many plasma membrane proteins' activity depends on neighbor lipids. For example, multidrug resistance pumps (MDR) need close contact with other components of the membrane in order to perform their regular transport activity. They also have different lipid affinities. Sterols are included in these membrane components and the studied ERG mutants accumulate bended, not flat, sterols, which along with sphingolipids might alter the efflux abilities of MDR. Another example is the inactivation of ATPases when delipidated. [85,86]

The most important sterol in yeasts is ergosterol, whose main source is biosynthesis when oxygen, as a cofactor of several enzymes, is available. Other sterols precursors normally have low concentration in the cell.[87] A schematic view of ergosterol synthesis and the enzymes' functions are shown in *Figure 2* and *Figure 3*, respectively. Ergosterol is associated with growth's and proliferation's incitement and with stress adaptation (such as, low temperatures, low concentration of sugars, resistance to alcohol, salt, drugs and lactone, oxidative stress and hypoxic response, among others). Ergosterol has several cellular functions and each is carried out when ergosterol is in a specific range of concentration. This means that ergosterol proportion should be appropriately regulated in order to keep a correct level in cells.

Many studies have been done to assess the role of ergosterol in stress adaptation.[88] For example, a study showed that complete blockage of the ergosterol biosynthesis pathway by drugs or mutation of its genes leads to diminished drug, salt, lactones and oxidative stress tolerance. [88,84,89] Ergosterol productivity can be increase by overexpressing certain biosynthesis genes (*ERG1*, *ERG4*, *ERG9* and *ERG11*).[88,90,91] Even though complete blockage of ergosterol biosynthesis leads to stress sensitivity, including osmotic and salt stress, upon this stress the yeast does not actually increase its ergosterol content. On the contrary, ERG genes are repressed leading to lower ergosterol during adaptation to hyperosmotic, salt or oxidative stress.[89] This process is controlled by transcription factor *MOT3* and *ROX1* and by HOG1 MAP kinase.

A study showed that overexpression of *HMG1*, *HMG2*, *ERG1*, *ERG4*, *ERG5*, *ERG6*, *ERG7*, *ERG9*, *ERG10*, *ERG11*, *ERG13*, *ERG19*, *ERG20*, *ERG24*, *ERG25*, *ERG26*, *ERG27*, *ERG28* or *NCP1* resulted in slower growth when compared to wild-type. In this study, they started by observing that total ergosterol concentration decreased significantly when exposed to hyperosmotic and salt stress (> 0.7 M sodium chloride or > 1 M sorbitol). Inhibitors of ergosterol biosynthesis (fluconazole or ketoconazole) were used to artificially decrease ergosterol concentration and resulted in earlier recovery from sodium chloride stress. This shows that inhibition of ergosterol synthesis is a growth advantage when yeast is in under salt stress.

Inability to inhibit ergosterol synthesis leads to hyper-accumulation of toxic sodium ions and hence to salt stress susceptibility. This hyper-accumulation may be due to a higher uptake of sodium ions or/and to a lower extrusion of sodium ions in the membrane. The membrane H^+-ATPase , Pma1p, and potassium ions uptake system, Trk, are the most relevant transporters in generation and consumption of membrane potential, respectively. Also, Na^+-ATPase, Ena1p, is the principal sodium ions extrusion system.[92,93]

Finally, they tested if over activation of ergosterol synthesis interfered with the processes above using a *UPC2-1* mutant. *UPC2-1* mutant has a mutation that makes the Upc2p transcription factor constitutively active and allows ergosterol uptake under oxygen presence (normally repressed). This mutant accumulated greater quantities of ergosterol than the wild-type I control and was unable to decrease ergosterol concentration when exposed to oxidative stress. *ERG2* and *ERG11* were over expressed constructively and independently on salt stress. In menadione stress, its growth was also highly inhibited, while in salt stress average concentrations of sodium or potassium ions were mote toxic than in the wild-type resulting in drastic susceptibility to this stress. Ergosterol synthesis inhibitors, such as fluconazole and growth in high potassium ions, reverted this hypersusceptibility to salt stress. The mutant was also sensitive to other toxic cations: tetramethylammonium, norspermine and hygromycin b. Addition of external sterol under salt stress deepened sensitivity to the stress. They concluded that the mutant resulted in constitutive and stress-independent over expression of ERG genes with excessive accumulation of ergosterol and serious sensibility to salt and oxidative stress. This sensitivity was augmented by addition of external ergosterol and amended by ergosterol synthesis inhibitors.

In conclusion, ERG genes are repressed leading to lower ergosterol content in the cell when the yeast is adapting to hyperosmotic, salt or oxidative stress. Also, *ERG2* and *ERG11* are specifically targeted under salt stress. If the yeast is unable to inhibit ergosterol synthesis, there is hyper-accumulation of toxic sodium ions and hence to salt stress susceptibility, which might be due to a higher uptake of sodium ions or/and to a lower extrusion of sodium ions in the membrane[89]

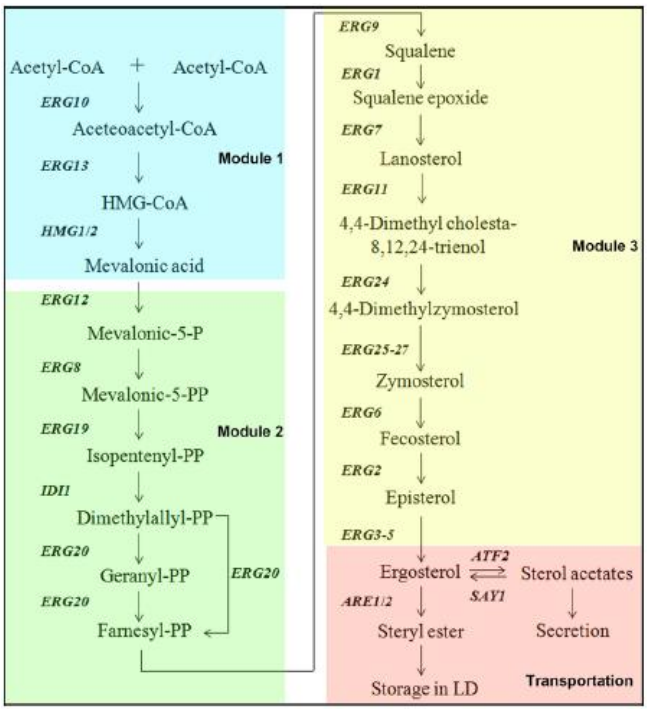


Figure 2: Schematic view of ergosterol synthesis

Gene	Gene ID	EC number	Function
Mevalonate biosynthesis			
ERG10	YPL028W	EC:2.3.1.9	Acetoacetyl-CoA thiolase
ERG13	YML126C	EC:2.3.3.10	Hydroxymethylglutaryl-coenzyme A synthase
HMG1	YML075C	EC:1.1.1.34	Hydroxymethylglutaryl-coenzyme A reductase
HMG2	YLR450W	EC:1.1.1.34	
Farnesylpyrophosphate biosynthesis			
ERG12	YMR208W	EC:2.7.1.36	Mevalonate kinase
ERG8	YMR220W	EC:2.7.4.2	Phosphomevalonate kinase
ERG19	YNR043W	EC:4.1.1.33	Diphosphomevalonate decarboxylase
IDI1	YPL117C	EC:5.3.3.2	Isopentenyl diphosphate isomerase
ERG20	YJL167W	EC:2.5.1.10	Polyprenyl synthetase
Ergosterol iosynthesis			
ERG9	YHR190W	EC:2.5.1.21	Squalene synthetase
ERG1	YGR175C	EC:1.14.13.132	Squalene epoxidase
ERG7	YHR072W	EC:5.4.99.7	lanosterol cyclase/lanosterol synthase
ERG11	YHR007C	EC:1.14.13.70	Cytochrome P450 lanosterol 14a-demethylase
ERG24	YNL280C	EC:1.3.1.70	Sterol C-14 reductase
ERG25	YGR060W	EC:1.14.13.72	C-4 methyl sterol oxidase
ERG26	YGL001C	EC:1.1.1.170	Sterol C-4 decarboxylases
ERG27	YLR100W	EC:1.1.1.270	3-Keto-steroid reductase
ERG6	YML008C	EC:2.1.1.41	C-24 sterol methyltransferase
ERG2	YMR202W	EC:5.3.3.5	C-8 sterol isomerase
ERG3	YLR056W	EC:1.3.3.-	C-5 sterol desaturase
ERG5	YMR015C	EC:1.14.-.-	C-22 sterol desaturase
ERG4	YGL012W	EC:1.3.1.71	C24 (28) sterol reductase

Figure 3:Enzyme's corresponding gene and function

Materials and Methods

Strain and media

S. cerevisiae strain BY4741, obtained from the Euroscarf collection, and *S. boulardii* strain CNCM I-745, isolated from a ULTRA-LEVURE®'s sachet (Biocodex, Beauvais, France), were stored in YPD medium. Depending on the essay, each of the two strains was cultivated in SIEM liquid medium (pH 7)[94,95] or in modified YPD liquid medium. The composition of the SIEM and YPD liquid media can be found in *Table 6* and *Table 7*, respectively, while data concerning the used enzymes can be found in *Table 8*. Cell growth was analyzed by measuring the optical density (OD_{600nm}) of the cellular suspensions at 600 nm.

Table 6: SIEM liquid medium's composition (initial version).

Substance		Concentration (g/L)	Enzymatic activity (U/L)
BD Bactotryptone		5.7	-
D-glucose		2.4	-
Salts, excluding bile salts	Sodium chloride	6.14	-
	Monopotassium phosphate	0.68	-
	Monosodium phosphate	0.3	-
	Sodium bicarbonate	1.01	-
Bile salts	Sodium cholate	2.8	-
	Sodium deoxycholate	2.8	-
Enzymes	Lysozyme	0.2	-
	α-amylase	-	1000
	Trypsin	-	110
	Chymotrypsin	-	380
	Lipase	-	960

Table 7: Modified YPD liquid medium composition.

Substance		Concentration (g/L)	Enzymatic activity (U/L)
BD Bactopeptone		20	-
D-glucose		20	-
Yeast extract		10	
Salts, excluding bile salts	Sodium chloride	6.14	-
	Monopotassium phosphate	0.68	-
	Monosodium phosphate	0.3	-
	Sodium bicarbonate	1.01	-
Bile salts		2.8	-
		2.8	-
Enzymes	Lysozyme	0.2	-
	α-amylase	-	1000
	Trypsin	-	110
	Chymotrypsin	-	380
	Lipase	-	960

Table 8: Enzymatic data, specific activity, solubility, purity, storage and dissolution method for each enzyme.

Enzyme	Specific activity (U/mg)	Solubility (mg/mL)	Purity (%)	Storage method	Dissolve in
Lysozyme	40000	10	98	Solution (2 a 8 ºC)	Water
α-amylase	1500	0.1	30	Solution (2 a 8 ºC)	Water
Trypsin	1500	4	100	Freeze (-20ºC)	Hydrochloric acid (1 mM)
Chymotrypsin	40	2	85	Freeze (-20ºC)	Hydrochloric acid (1 mM)
Lipase	250	0.1	20	Solution (2 a 8 ºC)	Water

Total RNA extraction and RNA sequencing

Three replicates of *S. cerevisiae* and of *S. boulardii* were grown in SIEM liquid medium with half that salts (sodium chloride, 3.07 g/L; monopotassium phosphate, 0.34 g/L; monosodium phosphate, 0.15 g/L and sodium bicarbonate, 1.00 g/L) and bile salts (1.4 g/L each), until early-log phase (OD_{600nm} of 0.8±0.05). Cells were harvested by centrifugation for 5 minutes at 7000 rpm and 4ºC and washed twice with distilled water. The resulting pellets were stored at -80ºC. Total RNA was isolated using an Ambion Ribopure-Yeast RNA kit, according to manufacturer's instructions.

Prior to RNA-seq analysis and for library preparation, quality control measures were implemented. Concentration of RNA was ascertained via fluorometric analysis on a Thermo Fisher Qubit fluorometer. Overall quality of RNA was verified using an Agilent Tapestation instrument. Following initial QC steps sequencing libraries were generated using the Illumina Truseq Stranded Total RNA library prep kit with ribosomal depletion via RiboZero Gold according to the manufacturer's protocol. Briefly, ribosomal RNA was depleted via pull down with bead-bound ribosomal-RNA complementary oligomers. The RNA molecules were then chemically fragmented and the first strand of cDNA was generated using random primers. Following RNase digestion the second strand of cDNA was generated replacing dTTP in the reaction mix with dUTP. Double stranded cDNA then underwent adenylation of 3' ends following ligation of Illumina-specific adapter sequences. Subsequent PCR enrichment of ligated products further selected for those strands not incorporating dUTP, leading to strand-specific sequencing libraries. Final libraries for each sample were assayed on the Agilent Tapestation for appropriate size and quantity. These libraries were then pooled in equimolar amounts as ascertained via fluorometric analyses. Final pools were absolutely quantified using qPCR on a Roche LightCycler 480 instrument with Kapa Biosystems Illumina Library Quantification reagents.

Strand specific RNA-seq library preparation and sequencing was carried out as a paid service by Somagenics Inc., Santa Cruz, California, USA. Paired-end reads (Illumina NextSeq 500 v2, 2x150 bp, 2 GB clean data). Obtained reads for each sample yielded 1.51 to 3.94×10^7 reads for BY4741 samples and 5.15 to 6.19×10^7 reads for Biocodex samples. Three replicates of each sample were obtained from three independent RNA isolations. After receiving the results, samples reads were trimmed using Skewer [96] and aligned to the *S. cerevisiae* reference genome, obtained from the *Saccharomyces* Genome Database (CGD) (http://www.yeastgenome.org/), using TopHat [97]. HTSeq [98] was used to count mapped reads per gene. Differentially expressed genes were identified using

DESeq2 [99] with an adjusted p-value threshold of 0.01 and a $\log_2$ fold change threshold of -1.0 and 1.0. Default parameters in DESeq2 were used.

Adhesion to human epithelial cell

FHs 74 Int small intestine cell line (ATCC® CCL-241™) was routinely maintained in Hybri-Care Medium (ATCC® 46-X) supplemented with 0.15% $NaHCO_3$, 30 ng/mL epidermal growth factor (EGF) and 10% of fetal bovine serum (FBS) in a humidified atmosphere at 37ºC with 5% CO_2. Cells were cultivated in 24-well polystyrene plates (Greiner), in Hybri-Care Medium (ATCC® 46-X™), until a density of $1.25x10^5$ cells/well was reached after 24h of incubation. The culture medium was then removed and substituted by fresh culture medium. *S. boulardii* and *S. cerevisiae* cells, were cultivated in YPD medium overnight, washed in PBS pH 7.4 buffer and added to each well with a density of $1.25x10^6$ cells/well (MOI=10). Plates were incubated at 37ºC, 5% CO_2 for 30 min. Each well was washed 3 times with 500 µL PBS pH 7.4, followed by the addition of 500 µL of Triton X-100 0.5% (v/v) and incubation at room temperature for 15 min to allow degradation of the human cells. The cell suspensions in each well was then recovered and spread onto YPD agar plates and incubated at 30ºC for 48h. CFU (Colony Forming Units) were counted for each well, representing the proportion of cells adherent to the human epithelium. Statistical analysis of the results was performed using analysis of variance and differences were considered significant for $p < 0.05$. This procedure was realized by Doctor Pedro Pais and results were included in this thesis for their significance in the present theme.

Results and discussion

Optimization of *in vitro* gastrointestinal tract-like growth media

In order to evaluate the global gene expression pattern in *S. boulardii* when compared with *S. cerevisiae*, cultivation conditions that mimic the gastrointestinal tract, but still enable the growth of both strains, were looked for. As starting point the SIEM liquid medium[94,95] was tested. Surprisingly, although *S. cerevisiae* did grow in this medium, *S. boulardii* was unable to do so (*Figure 4*).

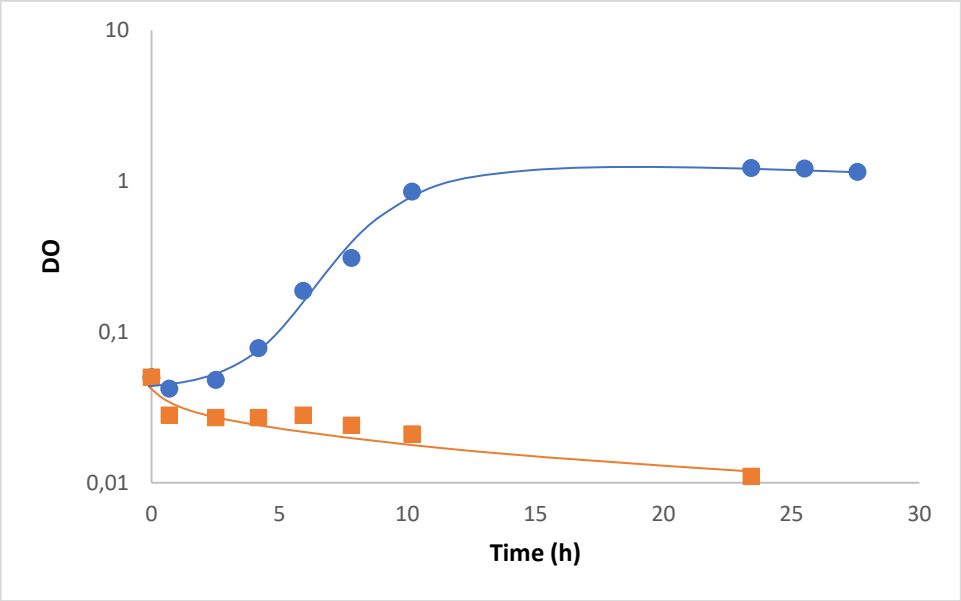


Figure 4: S. cerevisiae (●) and S. boulardii (■) growth curve in SIEM liquid medium.

To optimize the SIEM liquid medium to enable *S. boulardii* growth, several adaptations were tested. In *Table 9*, the 35 media tested are described. As a first hypothesis, it was considered that the gastrointestinal enzymes might inhibit the yeast's growth, but the results (medium 3a to 3f) showed that either the enzymes were not responsible for the inhibition or they were not the only ones responsible. The yeast continued not to grow even though the medium was supplemented with different sources of nutrients (ammonium sulphate, YNB, peptone instead of tryptone (medium 4a to 4c); yeast extract, higher concentration of glucose (medium 5a to 5c)). Supplementation with yeast extract and inhibition by enzymes was analyzed in medium 6a to 6f, but *S. boulardii* still didn't grow.

YPD liquid medium, supplemented with salts, bile salts and gastrointestinal enzymes, was also analyzed and *S. boulardii* still did not grow, so it was concluded that the lack of growth was not due to lack of nutrients but to inhibition by one or several of the media's constituents. Inhibition of each group of substances (salts, bile salts and enzymes) was tested by themselves or in combination (medium 8a to 8f and 9a to 9f). It was found that bile salts were responsible for growth inhibition (medium 8c, 8f, 9c and 9f). All media without bile salts were able to sustain cell growth, however non-bile salts partially inhibited growth, as we can also see in *Figure 5*.

The final optimized growth medium contained bile salts and non-bile salts, but their concentration was reduced to half of the initial concentration (intestinal like medium (ILM) media). It

was then possible to register growth of *S. boulardii* cells in a medium that includes all components of the gastro intestinal tract (*Figure 6*).

Table 9: 35 different media used to cultivate S. boulardii in a simulated intestinal environment and the results of the growth. Yes represents limited growth.*

Number of medium	Medium	Growth	
		S. cerevisiae	*S. boulardii*
1	Liquid SIEM medium (overnight)	Yes	No
2	Liquid SIEM medium (for 24h)	Yes	No
3 a	Liquid SIEM medium without enzymes	-	No
3 b	Liquid SIEM medium without α-amylase	-	No
3 c	Liquid SIEM medium without lysozyme	-	No
3 d	Liquid SIEM medium without trypsin	-	No
3 e	Liquid SIEM medium without chymotrypsin	-	No
3 f	Liquid SIEM medium without lipase	-	No
4 a	Liquid SIEM medium supplemented with 2.7 g/L of ammonium sulphate	-	No
4 b	Liquid SIEM medium supplemented with 2.7 g/L of ammonium sulphate and 1.7 g/L of YNB	-	No
4 c	Liquid SIEM medium without tryptone and supplemented with 5.7 g/L of peptone	-	No
5 a	Liquid SIEM medium supplemented with 5 g/L of yeast extract	-	No
5 b	Liquid SIEM medium with 5 g/L instead of 2.4 g/L of glucose	-	No
5 c	Liquid SIEM medium with 5 g/L instead of 2.4 g/L of glucose and supplemented with 5 g/L of yeast extract	-	No
6 a	Liquid SIEM medium supplemented with 5 g/L of yeast extract without enzymes	-	No
6 b	Liquid SIEM medium supplemented with 5 g/L of yeast extract without lysozyme	-	No
6 c	Liquid SIEM medium supplemented with 5 g/L of yeast extract without α-amylase	-	No
6 d	Liquid SIEM medium supplemented with 5 g/L of yeast extract without trypsin	-	No
6 e	Liquid SIEM medium supplemented with 5 g/L of yeast extract without chymotrypsin	-	No
6 f	Liquid SIEM medium supplemented with 5 g/L of yeast extract without lipase	-	No
7	Modified liquid YPD medium	-	No
8 a	Liquid SIEM medium without salts, bile salts nor enzymes	-	Yes
8 b	Liquid SIEM medium without bile salts nor enzymes	-	Yes*
8 c	Liquid SIEM medium without (non-bile) salts nor enzymes	-	No
8 d	Liquid SIEM medium without salts nor	-	Yes

(Continuação) Table 10: 35 different media used to cultivate S. boulardii in a simulated intestinal environment and the results of the growth. Yes represents limited growth.*

	bile salts		
8 e	Liquid SIEM medium without bile salts	-	Yes*
8 f	Liquid SIEM medium without (non-bile) salts	-	No
9 a	Modified liquid YPD medium without salts, bile salts nor enzymes (normal liquid YPD medium)	-	Yes
9 b	Modified liquid YPD medium without bile salts nor enzymes	-	Yes*
9 c	Modified liquid YPD medium without (non-bile) salts nor enzymes	-	No
9 d	Modified liquid YPD medium without salts nor bile salts	-	Yes
9 e	Modified liquid YPD medium without bile salts	-	Yes*
9 f	Modified liquid YPD medium without (non-bile) salts	-	No
10 a	ILM media		Yes
10 b	Modified liquid YPD medium with half of the salts and bile salts		Yes

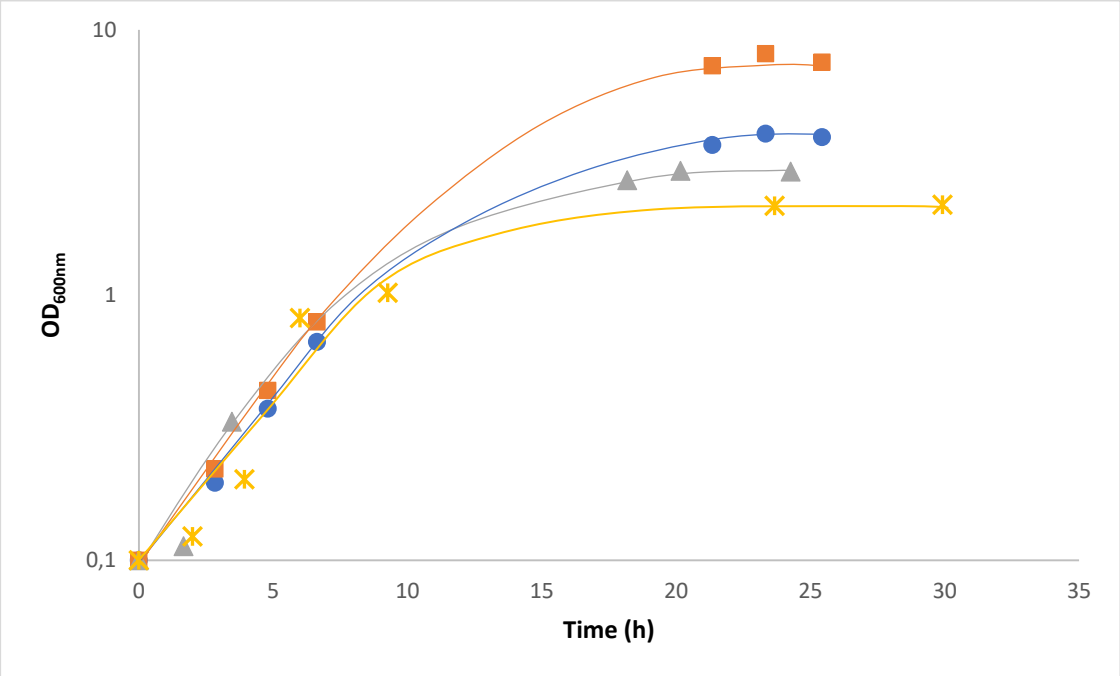


Figure 5: S. boulardii growth curve in SIEM liquid medium without salts, bile salts nor enzymes (●); SIEM liquid medium without salts nor bile salts (▲);modified liquid YPD medium without bile salts (■) and ILM medium (+).

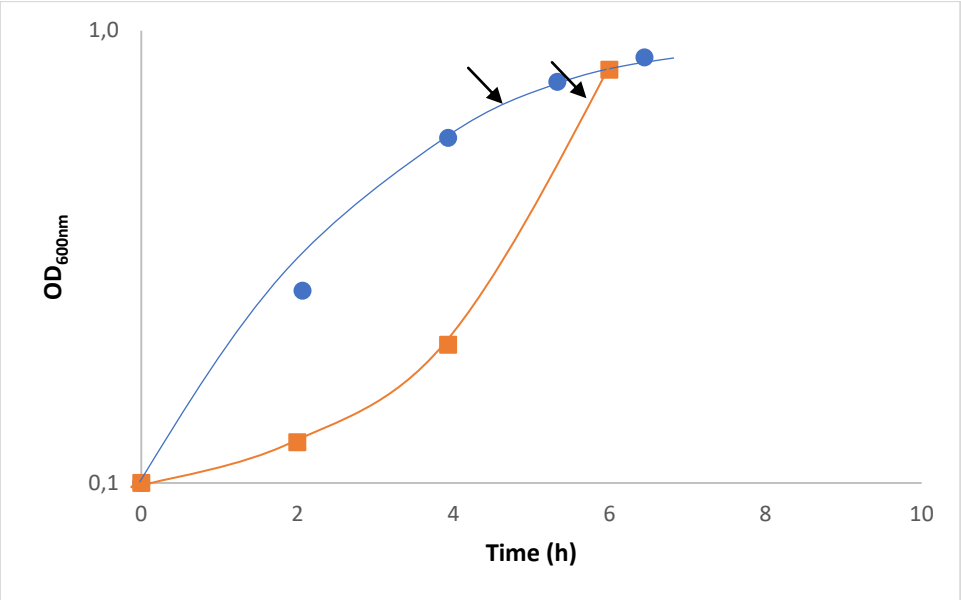


Figure 6: S. cerevisiae (●) and *S. boulardii* (■) growth curve in ILM medium. The arrow indicates the time point and OD_{600nm} (0.8, approximately) at which RNA extraction was done.

It was concluded that *S. boulardii* is sensitive to salt stress, especially to bile salts, when compared to *S. cerevisiae*. However this sensitivity does not necessarily correlate to cell survival, since *S. boulardii* has been shown to be haloduric, meaning that it can survive at high salt concentration, but is unable to grow. [79] Although *S. boulardii* was able to survive in the final optimized growth medium that contained half the bile salts and non-bile salts, it seems like this strain needs a higher period of adaptation than *S. cerevisiae* to the medium. However, both strains are eventually able to resume exponential growth, reaching similar maximum final biomass levels.

Global gene expression analysis

Once the growth conditions were optimized, the transcriptome of *S. cerevisiae* cells was compared to that of *S. boulardii* cells cultivated for about 6 hours in ILM medium. As mentioned above, the selected medium mimics the intestinal track environment, thus posing challenging conditions for both yeasts. Among the conditions that might be viewed as stressful by each yeast are high salt stress (especially bile salt stress), heat shock (especially for *S. cerevisiae*), enzyme activity (lysozyme, α-amylase, trypsin, chymotrypsin and lipase) and neutral pH.

Overall 577 and 523 genes were found to display higher and lower expression, respectively, in *S. boulardii*, when compared to *S. cerevisiae*. The list of genes was clustered according to functional categories, leading to the identification of biological functions that appear to be up- or down-regulated in *S. boulardii*, when compared to *S. cerevisiae* in the studied environment. Overall, the most overexpressed categories in *S. cerevisiae* were “Unknown function” and “Carbohydrate and energy metabolism”, while the most up-regulated categories in *S. boulardii* were “RNA metabolism and translation” and “Nitrogen and amino acid metabolism”. Smaller differences were also observed in the “Drug resistance”, “Oxidative stress” and “Protein degradation” functions which were found to be over-represented within the *S. cerevisiae* over-expressed genes; and in “Probiotic activity”, “Lipid

metabolism" and "Osmotic stress", functions which were found to be over-represented among the *S. boulardii* over-expressed genes (*Figure 7*).

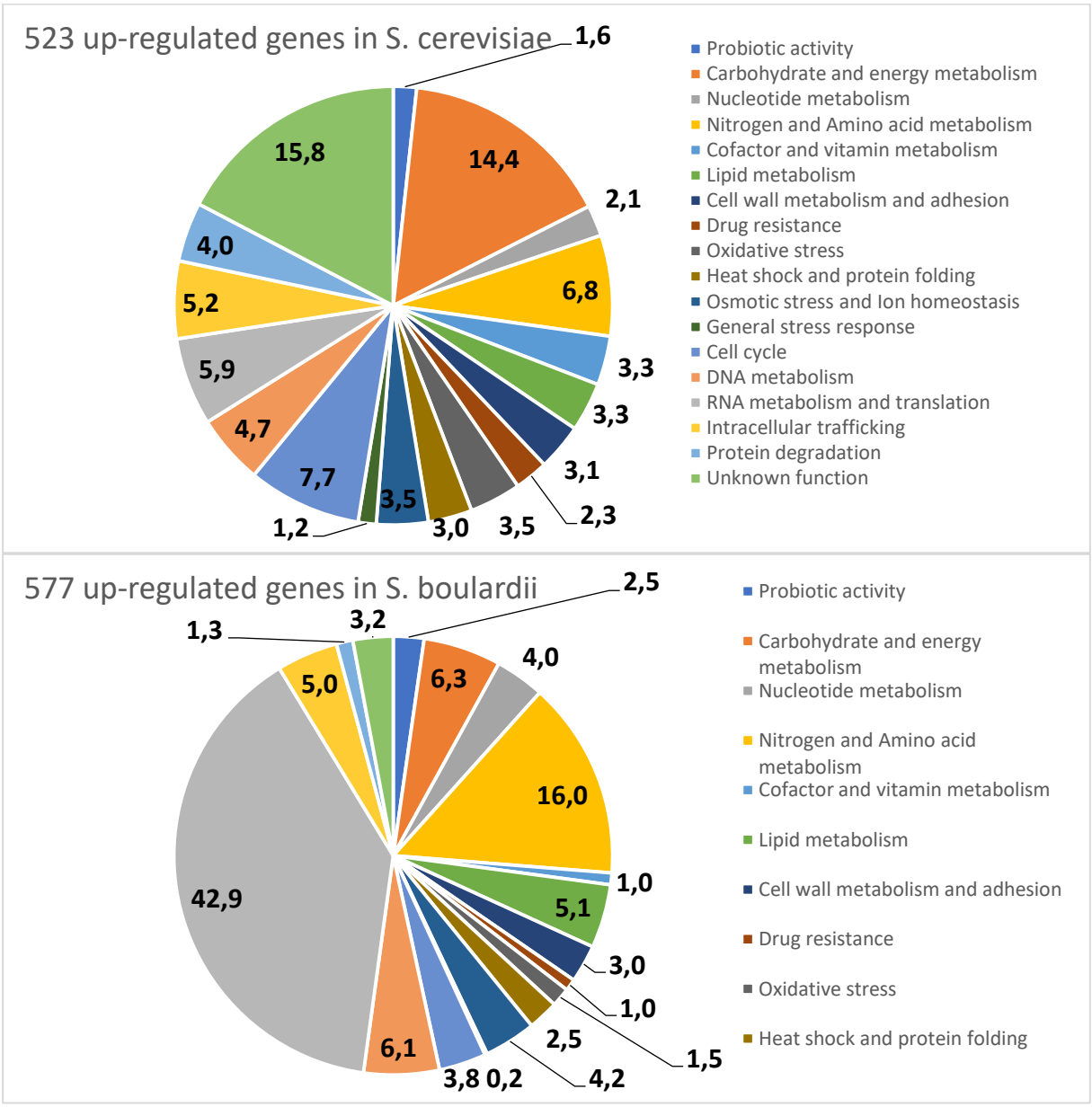


Figure 7: Main functional categories found to be over-expressed in S. cerevisiae or in S. boulardii.

The following subsections discuss the main differences in gene expression between the two species, highlighting roles with a potential impact in the probiotic activity of *S. boulardii*, namely in terms of the response to the components of the medium and the stress they may induce: general stress response, heat shock, salt stress response, and response to hydrolytic enzymes. Finally, the expression of genes that are likely to play a role in conferring probiotic properties is also analysed.

General stress response

As described in the Introduction of this thesis, there is a large set of genes whose expression changes in response to all stress conditions, irrespectively of the specific source of stress.

An overall look at the genes whose expression is up-regulated in *S. cerevisiae* when compared to *S. boulardii* appears to suggest that *S. cerevisiae* is feeling a lot more stress than *S. boulardii*, especially in terms of heat shock and oxidative stress.

In *Table 11* the general stress transcriptional response[73, 74] is compared to the genes whose expression was up or down-regulated in *S. cerevisiae* when compared with *S. boulardii*, respectively, in the selected intestinal tract mimicking medium. Overall, carbohydrate and energy metabolism (mitochondrial cellular respiration), oxidative stress, heat shock and protein degradation were all up-regulated in *S. cerevisiae* when compared with *S. boulardii*.

Indeed, many genes related to glycolysis (*GPM1*, *GPM2*, *MIG1*, *TYE7*, *ERR1*, *ERR2*, *ENO1*, *HXK1*, *GLK1*), pentose phosphate pathway and fructose and mannose metabolism (*PGI1*, *FBP1*, *FBA1*, *TDH2*, *TDH3*, *TDH1*, *ADH4*, *PGK1*, *GND1*, *TKL2*, *SOL4*, *XYL2*, *NQM1*, *DFS1*, *SOR2* and *TPI1*), TCA cycle (*PCK1*, *CIT3*, *SDH2*, *SDH3*, *SHH3*, *SHH4*, *LSC2* and *GRE2*), glyoxylate cycle (*CIT3*, *FDH1* and *YPL113C*), pyruvate (*HSP31*, *GLO4*, *CYB2*, *HSP32*, *SNO4*, *PDC6*, *PCK1* and *CDC19*) and glycogen (*GLG1*, *GSY2*, *GLC3*, *GPH1*, *GAC1*, *IGD1*, *SGA1* and *PIG2*) metabolism were found to be over-expressed in *S. cerevisiae*, when compared with *S. boulardii*, in cells grown in intestinal tract like medium. Also, genes required for mitochondrial respiration (*CYC1*, *COQ4*, *COQ9*, *COX5B*, *CYC7*, *QCR9*, *SDH2*, *SDH3* and *CYB2*), used as an energy source, are up-regulated in *S. cerevisiae* when compared with *S. boulardii*.

Significantly, genes involved in response to oxidative stress in mitochondria, in detoxifying reactive oxygen species and in general response to oxidative stress display up-regulated expression in *S. cerevisiae* when compared with *S. boulardii*. These include glutathione metabolism genes (*GTT1*, *GTT2*, *GPX1*, *IDP3*, *GND2*, *ECM4*, *GLO4* and *GRX2*), which represent a good part of the observed oxidative stress response, but also those encoding antioxidant enzymes (*GAD1*, *ALD2*, *GRE2*, *ALD3* and *SOD1*) and metabolic enzymes required for the production of reducing power, mainly through the regeneration of NADPH. Under stress, yeast cells try to maintain their internal redox potential by synthesizing and recycling redox buffer molecules like glutathione, or more directly by detoxifying reactive oxygen species, through the activity of superoxide dismutases and catalases. As it has already been said, pentose phosphate pathway, which is up-regulated, regenerates NADPH, which may help restore NADPH reducing equivalents for combating oxidative stress. Interestingly, oxidative stress genes are significantly more up-regulated in *S. cerevisiae* when compared with *S. boulardii*, when grown in the intestinal tract like medium, than in general stress response [73, 74], which suggests that the intestinal tract like medium used is perceived by *S. cerevisiae*, but not by *S. boulardii*, as a source of oxidative stress.

Another feature of the general stress response, the expression of genes related to protein degradation, was also found to be up-regulated in *S. cerevisiae*, when compared with *S. boulardii*. Degradation is necessary for proteins that cannot be refolded or that are damaged or denatured in order to avoid their aggregation. Targeting of proteins for turnover can be done by ubiquitination (ubiquitin ligation and conjugation, polyubiquitin and deubiquitinating enzyme). Ubiquitination followed by autophagy, vacuolar protein degradation or proteasomal degradation may accelerate the cells ability to change its protein internal repertory in response to stress conditions.

Additionally, the down-regulation of ribosome proteins, RNA metabolism, translation and amino acid synthesis, which is a significant part of the general stress response, is clearly observed in *S. cerevisiae*, when compared to *S. boulardii* in the selected growth medium. Under stress, it is believed that these processes are down-regulated, probably because these are very energy consuming and their repression saves energy that can be relocated to synthesis of molecular chaperones, protein degradation and other mechanism involved in stress response. Also, amino acid synthesis, including that of phenylalanine, tyrosine, tryptophan, histidine, arginine, proline, glycine, serine, threonine, valine, leucine, isoleucine, lysine, cysteine and methionine, was found to be down-regulated in *S. cerevisiae* when compared with *S. boulardii*. This is expected under stress, since protein synthesis is mostly repressed, while degradation of unfolded proteins is up-regulated [69–71,75,72,81].

Response to Heat shock stress

Overall, heat shock gene expression was found to be up-regulated in *S. cerevisiae* when compared with *S. boulardii*. Indeed, the expression of *SSA4*, *HSP82*, *HSP104*, *SSA3*, *HSP30*, *HSP12* and *HSP26* genes, among other genes encoding chaperones that help in protein conformation maintenance or refolding, was found to be over-expressed in *S. cerevisiae*, which is consistent with the notion that at 37ºC *S. cerevisiae* is suffering from heat shock, whereas *S. boulardii* is not. Synthesis of these chaperones and their activity consume lots of energy through ATP hydrolysis, which decreases ATP levels, affects central energy metabolism and leads to an increase in (or/and relocation of) energy production. Considering this effect, it is consistent to observe that together with the over-expression of HSP genes, carbohydrate and energy metabolism genes are also up-regulated in *S. cerevisiae*, when compared to *S. boulardii*, to an extent that appears far greater than the typical general response stress.

Table 11: Comparison of the general stress response genes from two studies[73,74] with the gene expression profile of S. cerevisiae versus S. boulardii in this thesis. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

General Stress Response		Overexpressed in *S. cerevisiae* when compared to *S. boulardii*			General Stress Response		Overexpressed in *S. boulardii* when compared to *S. cerevisiae*		
Remodeling of Yeast Genome Expression in Response to Environmental Changes[73]	Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes[74]				Remodeling of Yeast Genome Expression in Response to Environmental Changes[73]	Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes[9]			
Up-regulated in salt stress studies or in *S. cerevisiae* when compared to *S. boulardii*					**Down-regulated in salt stress studies or in *S. cerevisiae* when compared to *S. boulardii***				
Carbohydrate metabolism					**Carbohydrate metabolism**				
Glycolysis					**Glycolysis**				
YLR345W	*HXK1*	*GPM1*	*TYE7*	*ENO1*			*SFA1*		
HXK1	*GLK1*	*GPM2*	*ERR1*	*HXK1*			*SPI4*		
GLK1		*MIG1*	*ERR2*	*GLK1*					
Pentose Phosphate Shunt and fructose and manose metabolism					**Pentose Phosphate Shunt and fructose and manose metabolism**				
GND2	*PFK26*	*PGI1*	*ADH4*	*NQM1*			*GND1*		
NQM1	*FBP26*	*FBP1*	*PGK1*	*DFS1*			*PKF27*		
FBP26	*PGM2*	*FBA1*	*GND2*	*SOR2*			*TKL1*		
PFK26		*TDH2*	*TKL2*	*TPI1*			*SOL3*		
PGM2		*TDH3*	*SOL4*				*PGM1*		
		TDH1	*XYL2*						
TCA cycle					**TCA cycle**				
GRE3		*PCK1*	*SDH3*	*LSC2*			*IDP1*		
		CIT3	*SHH3*	*GRE2*			*ACC1*		
		SDH2	*SHH4*						
Glyoxylate cycle					**Glyoxylate cycle**				
		CIT3	*FDH1*	*YPL113C*			*ICL1*		
Xylulose fermentation					**Xylulose fermentation**				
XKS1	*XKS1*								
Trehalose metabolism					**Trehalose metabolism**				
PGM2 *TPS3*	*PGM2* *TSL1*	*ATH1*							
TPS1 *TSL1*	*TPS1* *NTH1*	*NTH2*							
TPS2 *NTH1*	*TPS2* *ATH1*								
Glycogen metabolism					**Glycogen metabolism**				
GSY2	*GLG1*	*GLG1*	*GPH1*	*SGA1*			*AAP1*		
GPH1	*GSY2*	*GSY2*	*GAC1*	*PIG2*					
	GLC3	*GLC3*	*IGD1*						
Glycerol metabolism					**Glycerol metabolism**				
GPD1 *GLO2*	*GPD1*	*GPD1*							
GPP2 *DAK1*		*GPD2*							
GLO1		*YIG1*							
Pyruvate metabolism					**Pyruvate metabolism**				
		HSP31	*HSP32*	*PCK1*			*DLD2*		
		GLO4	*SNO4*	*CDC19*					
		CYB2	*PDC6*						
Pyruvate metabolismo and Glyoxylate cycle					**Pyruvate metabolismo and Glyoxylate cycle**				
		DAL7					*MLS1*	*ACS2*	
Glycan metabolism					**Glycan metabolism**				
		OST6	*AMS1*				*MNN10*	*MNN1*	*PMT4*
		SMP3					*ALG3*	*MNN1*	*PMT2*
Inositol phosphate metabolism					**Inositol phosphate metabolism**				
		INM2	*PLC1*				*INO1*		
		INP54	*ARG82*						
Galactose and palatinose metabolism					**Galactose and palatinose metabolism**				
		GAL7	*GAL1*	*MAL12*			*SUC2*	*IMA1*	*PGM1*

Continuation: Table 12: Comparison of the general stress response genes from two studies[73,74] with the gene expression profile of S. cerevisiae versus S. boulardii in this thesis. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

Hexose transport						**Hexose transport**				
	HXT5		HXT1	MAL11				HXT4		
			HXT10	SNF3						
Energy metabolism: mitochondrial cellular respiration						**Energy metabolism: mitochondrial cellular respiration**				
CYC7			CYC1	COX5B	SDH2			PMA1		
COX5B			COQ4	CYC7	SDH3					
			COQ9	QCR9	CYB2					
Stress response						**Stress response**				
Heat shock (see protein folding too)						**Heat shock (see protein folding too)**				
			MHO1	HSP30	SPG4			NAT1	SAM1	
			NNR2	SYM1	RPI1			SKN7		
			ECM10	GAC1	YGP1			SSB2		
			SPG1	NTH2				MGA1		
Oxidative stress						**Oxidative stress**				
Mitochondria						**Mitochondria**				
MCR1	SCO2	CCP1	CCP1					POS5		
CTT1		PRX1	PRX1					OGG1		
Glutathione						**Glutathione**				
GTT1		GPX1	GTT1	IDP3	GLO4			OPT1	RNR4	GND1
		GTT1	GTT2	GND2	GRX2			SFA1	IDP1	
			GPX1	ECM4				RNR3	GSH1	
Detoxifying reactive oxygen species						**Detoxifying reactive oxygen species**				
DAK1		SOD1	GAD1	GRE2	SOD1					
		CTT1	ALD2	ALD3						
Other						**Other**				
TTR1	DDR48	TRX2	TRX2	TSA2	RCK1			TAH18		
YCL035C	NCE103	TTR1	NCA3	MRX2	OXR1			GDN1		
TRR2		ECM38	BDH2	FBA1	LOT6			YHB1		
TSA2		TSA2	YML131W	RNY1				UGA3		
Osmotic stress						**Osmotic stress**				
			DOG2	MPC3	SIP18			SKO1		
			GPD1	GRE1						
			YML131W	GRE2						
Salt stress						**Salt stress**				
								ISC1	YGK3	
Ion homeostasis						**Ion homeostasis**				
BSD2			VHS3	LDH1		PKR1		SPF1	FRE1	AFT1
PPZ2			VMA22	IZH4		RAV2		PER1	FRE4	PMA1
			PMA2	PRM6		AST2		PCA1	ATX2	PKR1
			YKE4	RAV2				CCC2	CTR3	
			ZRT1	AST2				PMR1	TOM5	
			ALR2					GGC1	RSN1	
			ARN2					CTR1	FTR1	
			FIT2					MRS3	YMR279C	
Cell wall stress						**Cell wall stress**				
			HSP12	USV1				WSC2	NCW2	
Overall stress						**Overall stress**				
			MSC1	PFA3	SSA3			CGR1		
			FMP40	MAF1	MSN4			MRC1		
			FMP16	ALD2	SED1			CMK2		
			UGX2	ALD3	YRO2					
			MBR1	MRK1	YDR034W-B					
			DCS2	GPH1						
Drug Resistance						**Drug Resistance**				
			PDR3	YLR046C	ARR1			SNQ2	PBL2	
			PDR8	RDS1	TAT1			SUP45	IMD2	
			CAD1	YPC1	CRG1			FAP1	YLR179C	
			PDR11	PAD1	YNR064C			KRE33	SSZ1	
			MIG3	QDR1	QDR2			RPS14B	SRO9	

Continuation: Table 13: Comparison of the general stress response genes from two studies[73,74] with the gene expression profile of S. cerevisiae versus S. boulardii in this thesis. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

DNA damage					**DNA damage**		
		HSM3	*PCD1*				*HUG1*
Starvation					**Starvation**		
		SUT1	*SPL2*	*GSY2*			*ZPR1*
		XBP1	*SIP2*	*SNO4*			*PUB1*
		NPP2	*DSE1*	*YGP1*			*NRP1*
Other stress					**Other stress**		
		RTA1	*TIR1*				*PUG1*
		RIM13	*TPK2*				*PDR12*
Protein folding (chaperones and others)					**Protein folding (chaperones and others)**		
HSP12	*HSP26*	*HSP12*	*OPI10*				*ZUO1* Related to assembly of
HSP26	*HSP42*	*HSP26*	*FES1*				*HSP10* actin and tubulins
HSP42	*HSP 78*	*HSP82*	*TRX2*				*NMA111* *CCT8*
HSP78	*HSP104*	*HSP104*	*SIS1*				*SSB1* *CCT4*
HSP104	*SSA4*	*SSA4*					*KAR2* *CCT5*
SSA4	*SSA3*	*SSA3*					*SEC63* *CCT3*
SSE2	*SSE2*	*TRX3*					*CCT7*
Transport and catabolism & Protein degradation					**Transport and catabolism & Protein degradation**		
Protein degradation (proteosome)					**Protein degradation (proteosome)**		
PHB2 *YPS6*	*PRC1*	*ECM29*	*RPN14*	*VID24*			*CYM1*
SLT2 *RPN5*	*PAI3*	*YPS6*	*RPN13*	*NNK1*			*MNL1*
Vacuolar protein degradation					**Vacuolar protein degradation**		
	PMC1 *LAP4*	*COG7*					*CPS1*
	VAB31 *PEP4*	*SNA3*					*FRD1*
	PRB1 *PBI2*	*ATG34*					*VID22*
Protein degradation (ubiquitiination)					**Protein degradation (ubiquitiination)**		
UBC5	*UBC5*	*HEL1*	*UBC8*	*DIA2*			*NPR1*
UBC8	*UBC8*	*HRT3*	*UBC12*	*RRI1*			
	UBI4	*UBP9*	*SAF1*	*ULP2*			
	UBP15	*UBP7*	*FYV10*	*UBP11*			
		UBP8	*PIB1*				
Autophagy and Mitophagy					**Autophagy and Mitophagy**		
AUT1	*APG7*	*ATG31*	*ATG10*	*ATG34*			*ATG5*
	AUT7	*ATG17*	*ATG9*	*TPK2*			*MDM38*
	APG1	*ATG3*	*ATG32*	*MSN4*			*ATG1*
		ATG29	*STF1*				*UME6*
		INH1	*ATG36*				

Response to hydrolytic enzymes

The selected medium contained the following hydrolytic enzymes, typically found in the intestinal tract: lysozyme, α-amylase, trypsin and lipase.

Lysozyme is an antimicrobial peptide that hydrolyzes β-linkage between the N-acetylglucosamine and N-acetylmuramic of peptidoglycan present in gram-positive bacteria cell wall. This leads to cell lysis and death in a hypoosmotic environment. However, the yeast cell wall is not composed by peptidoglycan, but rather by mannoproteins, fibrous β(1→3) and β(1→6) glucans and chitin. Hence, lysozyme is unable to lyse yeast cells in the same way it does to gram-positive bacteria cells.

α-amylase is an enzyme that hydrolyses starch and glycogen into glucose and maltose. This includes hydrolysis of α(1-4) glycosidic bonds of exopolysaccharides present in extracellular polymeric substance secreted by bacteria to form biofilms. Hence, α-amylase is capable of degrading bacterial biofilms. Since yeast biofilms rely on a polymeric extracellular matrix composed by polysaccharides, it is possible that α-amylase might also be able to disrupt yeast biofilms. Since, in this thesis, biofilms were not analyzed in an intestinal simulated media, this disruption could not be evaluated. However, this enzyme may also be able to degrade chitin, when exposed. Usually, chitin is well protected by the rest of the cell wall above it, but during stress conditions it is possible that the protection offered by the cell wall is less effective and the enzyme might be able to affect chitin.

Trypsin is a serine protease that hydrolysis peptide chains in their lysine or arginine residues. Hence, this enzyme can possibly affect adhesion and flocculation proteins present in the surface of the yeast cell wall. Trypsin and α-chymotrypsin are tolerated by many cell types and, more specifically, do not seem to lyse or significantly affect *S. cerevisiae*'s cell wall even when used at the same time as glucanases. Although they do not seem to significantly affect cell viability, trypsin may still have an effect in external proteins, including those involved in functions such as flocculation, adhesion, antigen activation, recognition of mating partners, biofilm formation, pseudohyphal growth, iron retainment, uptake of sterols and cell wall hydrophobicity, synthesis and repair.

Lipase is an enzyme that hydrolyzes lipids, normally in their glycerol backbone. Yeast plasma membrane is mostly composed by lipids that are potential targets for lipases, it is protected by the cell wall. However, during stress conditions it is possible that the protection offered by the cell wall is less effective and this enzyme might affect the different lipids that compose the plasma membrane (mostly, phospholipids or glycerophospholipids). Additionally, bile salts as detergent like molecules that can also affect the membrane lipid bilayer of yeasts. Since the membrane is involved in Na^+, K^+ and pH homeostasis, nutrient and enzyme transport, mating, signal transduction, cytoskeleton anchoring, drug efflux, stress response, adhesion molecules and antigen activation, the presence of both lipase and bile salts in the GI tract medium can have strong repercussions at the level of plasma membrane related processes.

Altogether, the external cell wall proteins can be affected by both trypsin and α-chymotrypsin and, if the cell wall is disturbed during stress conditions and its protective effect is decreased, chitin

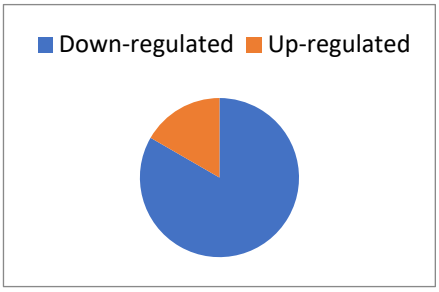
might be affected by lysozyme and α-amylase and plasma membrane lipids might be affected by lipase and bile salts. Thus, depending on their sensitivity to these enzymes, *S. boulardii* and *S. cerevisiae* may require specific changes in the transcription profile of cell wall and lipid metabolism related genes to adjust to them.

Indeed, lipid metabolism gene expression was found to be very different in *S. cerevisiae*, when compared to *S. boulardii*. Sphingolipids and fatty acid degradation were up-regulated (*Figure 8*), while ergosterol and glycerophospholipids metabolism were down-regulated in *S. cerevisiae*, when compared to *S. boulardii* (*Figure 9* and *Table* 14). Glycerophospholipids and ergosterol biosynthesis (**Erro! A origem da referência não foi encontrada.**) up-regulation and fatty acid degradation down-regulation in *S. boulardii* when compared to *S. cerevisiae* might be deployed to fight membrane stress due to lipase and bile acid effects, suggesting that *S. boulardii* is more sensitive to the effect of these stress agents than *S. cerevisiae*. This observation is consistent with the higher sensitivity exhibited by *S. boulardii* to bile salt concentration.

On the other hand, up-regulation of sphingolipids in *S. cerevisiae* can be due to a response to heat shock. Indeed, inositol-phosphate, ceramide and phytosphingosine biosynthesis are all mostly up-regulated in *S. cerevisiae* when compared with *S. boulardii*.

Table 14: Lipid metabolism affected genes, their description and fold change. Sphingolipids and fatty acid degradation were up-regulated, while ergosterol and glycerophospholipids metabolism were down-regulated in S. cerevisiae, when compared to S. boulardii

Metabolism	Gene ID in *S. cerevisiae*	Gene ID in *S. boulardii*	Gene name	(Log 2) Fold change	Description
Ergosterol	*YNR019W*	*KO01_04405*	*ARE2*	2,84	Acyl-CoA:sterol acyltransferase
	YMR202W	*KO01_03799*	*ERG2*	1,61	C-8 sterol isomerase
	YGL001C	*KO01_01919*	*ERG26*	1,37	C-3 sterol dehydrogenase
	YHR072W	*KO01_02259*	*ERG7*	1,33	Lanosterol synthase
	YGL012W	*KO01_01929*	*ERG4*	1,43	C-24(28) sterol reductase
	YHR007C	*KO01_02194*	*ERG11*	1,64	Lanosterol 14-alpha-demethylase
	YGR175C	*KO01_01761*	*ERG1*	1,49	Squalene epoxidase
	YNL111C	*KO01_04287*	*CYB5*	1,88	Cytochrome b5, involved in the sterol and lipid biosynthesis pathways
Glycerophospholipids	*YMR006C*	*KO01_03979*	*PLB2*	2,48	Phospholipase B (lysophospholipase)
	YNL169C	*KO01_04237*	*PSD1*	1,38	Phosphatidylserine decarboxylase of the mitochondrial inner membrane
	YNL130C	*KO01_04271*	*CPT1*	1,50	Cholinephosphotransferase
	YBR029C	*KO01_00326*	*CDS1*	1,16	Phosphatidate cytidylyltransferase (CDP-diglyceride synthetase)
	YDL052C	*KO01_00764*	*SLC1*	1,18	1-acyl-sn-glycerol-3-phosphate acyltransferase
	YDR123C	*KO01_00924*	*INO2*	2,27	Component of the Ino2p/Ino4p transcription activator required for derepression of phospholipid biosynthetic genes in response to inositol depletion
Sphingolipids	*YBR183W*	*KO01_00187*	*YPC1*	-1,03	Alkaline ceramidase
	YDR297W	*KO01_01076*	*SUR2*	-1,67	Sphinganine C4-hydroxylase
	YKL008C	*KO01_03035*	*LAC1*	-1,28	Ceramide synthase component
Fatty acid degradation	*YOR317W*	*KO01_04532*	*FAA1*	1,23	Long chain fatty acyl-CoA synthetase
	YER015W	*KO01_01471*	*FAA2*	-1,39	Medium chain fatty acyl-CoA synthetase
	YGL205W	*KO01_02093*	*POX1*	-1,41	Fatty-acyl coenzyme A oxidase, involved in the fatty acid beta-oxidation pathway
	YLR284C	*KO01_03519*	*ECI1*	-2,06	Peroxisomal Δ3, Δ 2-enoyl-CoA isomerase, hexameric protein, essential for the beta-oxidation of unsaturated fatty acids



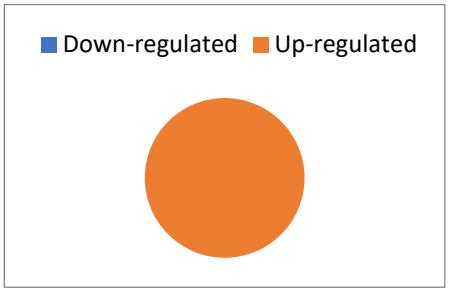


Figure 8: Comparison of up and down-regulated genes in S. boulardii when compared with S. cerevisiae for sphingolipids synthesis and fatty acid degradation

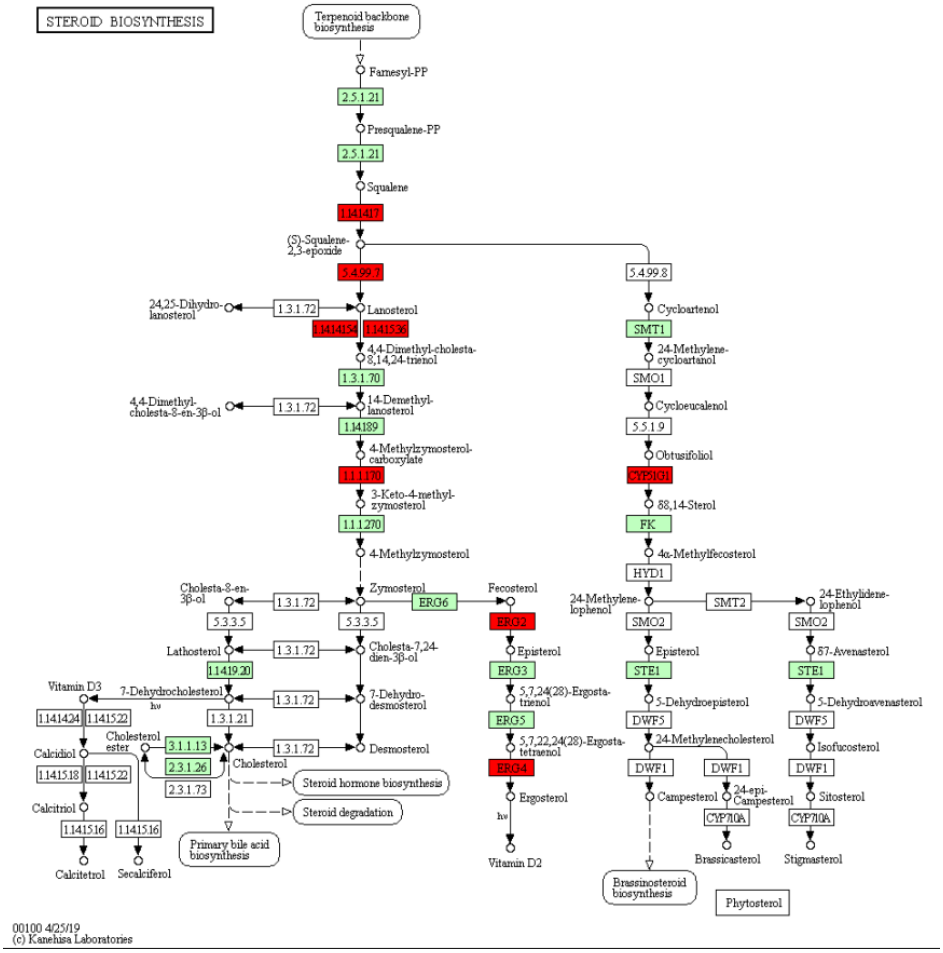


Figure 9: Comparison of up and down-regulated genes in *S. boulardii* when compared with s. cerevisiae for ergosterol and glycerophospholipids synthesis.In red are genes up-regulated in *S. boulardii* when compared with *S. cerevisiae*. In green are genes present in *S. cerevisiae* and similar yeast, while in white are genes not present.

Salt stress response

In this work, yeasts were subjected, among other things, to high levels of salt stress for a long period of time. It is then to be expected that the results will correlate better with studies that analyze later stages of salt stress (*Table 15*). The transcriptional response in salt stress occurs in the early stages of exposure in a transient manner, which means that many of these responses may be missed if the yeast is exposed to salt stress for too long, as was the case of this study. However, there are normally a high number of up-regulated genes for severe salt stress in later stages of stress exposure.

Osmotic stress may be balanced by adjusting the intracellular concentration of both osmolytes, such as glycerol or trehalose [69–71,72, 74,23,24], and of small ions such as Na^+, K^+ and H^+. Interestingly, most of the osmotic stress related genes showed higher expression in *S. cerevisiae*, when compared with *S. boulardii*. Indeed, the genes involved in the first steps of glycerol biosynthesis (*GPD1* and *GPD2*) were found to be up-regulated in *S. cerevisiae* when compared with *S. boulardii*. Glycerol synthesis and intracellular accumulation is increased during osmotic stress in order to adjust osmotic pressure along the cell membrane. By accumulating intracellularly, glycerol increases the uptake of water and consequently the cell swells to a certain size. This helps to fight water deficit and cell shrinkage and helps the yeast to restart growth. Additionally, glycerol is able to preserve the plasma membrane integrity and stabilize proteins, thus being a stress protectant, just like trehalose. Indeed, just like with trehalose, glycerol can be used as a compatible solute to counterbalance osmotic pressure. [69–71,79,72,80,75]

Altogether, the obtained results suggest that *S. cerevisiae* appears to be feeling more osmotic stress under ILM medium. Since apparently *S. cerevisiae* was found to grow better than *S. boulardii* in the presence of high salt concentrations, it is reasonable to hypothesize that it is the apparently higher ability to respond to osmotic stress that enables *S. cerevisiae* to survive in such conditions, when *S. boulardii* cannot.

Table 15: Comparison of several salt stress and osmotic transcriptional analysis studies with this thesis results. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

Salt stress				Overexpressed in *S. cerevisiae* when compared to *S. boulardii*			Salt stress				Overexpressed in *S. boulardii* when compared to *S. cerevisiae*
Physiological and transcriptomic analysis of a salt-resistant *Saccharomyces cerevisiae* mutant obtained by evolutionary engineering[72]	The Transcriptional Response of Yeast to Saline Stress[69]	Transcript Expression in *Saccharomyces cerevisiae* at High Salinity[71]	The Transcriptional Response of *Saccharomyces cerevisiae* to Osmotic Shock[81]				Physiological and transcriptomic analysis of a salt-resistant *Saccharomyces cerevisiae* mutant obtained by evolutionary engineering[72]	The Transcriptional Response of Yeast to Saline Stress[69]	Transcript Expression in *Saccharomyces cerevisiae* at High Salinity[71]	The Transcriptional Response of *Saccharomyces cerevisiae* to Osmotic Shock[81]	
Up-regulated in salt stress studies or in *S. cerevisiae* when compared to *S. boulardii*							**Down-regulated in salt stress studies or in *S. cerevisiae* when compared to *S. boulardii***				
Carbohydrate metabolism							**Carbohydrate metabolism**				
Glycolysis							**Glycolysis**				
UGP1 *HXK1* *GLK1* *GPM2* *EMI2*	*HXK2* *GLK1*		*UGP1* *YLR345W* *GLK1*	*GPM1* *GPM2* *MIG1* *TYE7* *ERR1*	*ERR2* *ENO1* *GLK1* *HXK1*						*SFA1* *SPI4*
Pentose Phosphate Shunt and fructose and manose metabolism							**Pentose Phosphate Shunt and fructose and manose metabolism**				
TKL2 *SOL4* *PGM2*		*ZWF1*	*GND2* *TKL2* *NQM1* *PGM2*	*PGI1* *FBP1* *FBA1* *TDH2* *TDH3* *TDH1*	*GND2* *TKL2* *SOL4* *XYL2* *ADH4* *PGK1*	*NQM1* *DFS1* *SOR2* *TPI1*					*GND1* *PKF27* *TKL1* *SOL3* *PGM1*
TCA cycle							**TCA cycle**				
			GRE3 *GRE2*	*PCK1* *CIT3* *SDH2*	*SDH3* *SHH3* *SHH4*	*LSC2* *GRE2*					*IDP1* *ACC1*
Glyoxylate cycle							**Glyoxylate cycle**				
				CIT3	*FDH1*	*YPL113C*					*ICL1*
Xylulose fermentation							**Xylulose fermentation**				
XKS1			*XKS1*								
Trehalose metabolism							**Trehalose metabolism**				
UGP1 *PGM2* *TPS1* *TPS2* *TSL1* *NTH1* *ATH1*	*TPS1* *TPS3* *TSL1* *NTH1*	*TPS1* *TPS2*	*UGP1* *PGM2* *TPS1* *TPS2* *TSL1* *NTH1*	*ATH1* *NTH2*							
Glycogen metabolism							**Glycogen metabolism**				
UGP1 *GLG1* *GSY1* *GSY2* *GLC3* *GPH1* *GDB1* *GAC1*	*PGM1* *UGP1* *GLG1* *GSY1* *GSY2* *GLC3* *GPH1* *GAC1*		*GSY1* *GSY2* *GLC3*	*GLG1* *GSY2* *GLC3* *GPH1* *GAC1* *IGD1* *SGA1* *PIG2*							*AAP1*

Continuation: Table 16: Comparison of several salt stress and osmotic transcriptional analysis studies with this thesis results. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

MRK1	*PPH21* *GLC7*											
Glycerol metabolism												
GPD1	*GPD1* *GPD2* *DAK1*	*GPD1* *GPD2* *DAK1* *STL1*	*GPD1* *GPP1* *GPP2* *DAK1* *GLO1*	*GPD1* *GPD2* *YIG1*								
Pyruvate metabolism												
				HSP31 *GLO4* *CYB2*	*PCK1* *CDC19* *PDC6*	*HSP32* *SNO4*				*DLD2*		
Pyruvate metabolismo and Glyoxylate cycle												
				DAL7						*MLS1*	*ACS2*	
Glycan metabolism												
				OST6 *SMP3*	*AMS1*					*MNN10* *ALG3*	*MNN1* *MNN11*	*PMT4* *PMT2*
Inositol phosphate metabolism												
				INM2 *INP54*	*PLC1* *ARG82*					*INO1*		
Galactose and palatinose metabolism												
				GAL7	*GAL1*	*MAL12*				*SUC2*	*IMA1*	*PGM1*
Hexose transport												
HXT2 *HXT 5* *HXT 6* *HXT7* *HXT 15* *HXT 16* *HXT 17* *MALL11* *STL1* *MTH1* *EMI2*	*HXT1* *HXT5* *HXT7* *HXT10* *STL1*	*HXT1* *HXT5* *MTH1*	*HXT1* *HXT5* *STL1*	*HXT1* *HXT10* *MAL11* *SNF3*						*HXT4*		
Aminoacid metabolism												
Alanine, aspartate and glutamate												
			GAD1	*PAN6*	*FOX2*	*GAD1*	*ASN2*	*GLN1*		*ASN1*	*CPA2*	*ASN2*
Phenylalanine, tyrosine, tryptophan and histidine												
				ALD2 *ALD3* *BNA1* *BNA2* *BNA4*	*CTA1* *HPA3*			*PRS3*	*DPH5*	*TRP3* *ARO3* *ARO7* *ARO1* *ARO4*	*TRP2* *HIS5* *BNA3* *TRP4* *ARO2*	Histidine *HIS3* *HIS7* *HIS1* *HIS4*
Arginine and proline												
		PUT4	*PUT4* *CAR1*	*DUR1,2*	Degradation *CAR2*				*PRO2*	*PRO2* *ARG5,6*	*ARG3* *ARG7*	*ARG8*
Glycine, serine and threonine												
				GPM1 *GPM2* *TDA10*			*SER1*	*THR4* *SER3* *THR1*		*SER2* *GLY1* *THR1*	*SER3* *THR4*	Degradation *GCV2*
Valine, leucine and isoleucine												
		BAP2		Degradation *POT1*			*LEU1*			*LEU1* *LEU2* *LEU9*	*ILV6* *ILV2* *ILV3*	*LEU4* *TTT1*
Lysine												
									LYS21	*ACO2* *LYS4*	*LYS2* *LYS1*	

Continuation: Table 17: Comparison of several salt stress and osmotic transcriptional analysis studies with this thesis results. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

Cysteine and methionine							**Cysteine and methionine**						
									MET13 MET25	SAM1 MET6 MET25	SAH1 MRI1 SAM4 SAM2	SAM1 MET17 MET2 MET13	MET6 IRC7
Biosynthesis of multiple amino acids							**Biosynthesis of multiple amino acids**						
				GDH3 AGX1 ALT2				HOM2	SER33	HOM3 CYS4 SHM1	TRP5 ILV1 SER33 ADE4 ARG4	ARG1 AAT2 HOM3 HOM2 GSH1	SPE2 SPE3 CYS4 CYS3
Biosynthesis or degradation of multiple amino acids							**Biosynthesis or degradation of multiple amino acids**						
			CHA1 ARO10 ARO9	HFD1 CHA1			BAT1				ARO9 ARO10 ALD5	ARO8 BAT1	
Amino acid transport							**Amino acid transport**						
				AGC1 VBA2	GAP1 TAT1	BTN2		PUT4 GNP1			AVT1 ORT1	MUP3 BAP3	
Energy metabolism: mitochondrial cellular respiration							**Energy metabolism: mitochondrial cellular respiration**						
		CYC7	CYC7 CYB2	CYC1 COQ4 COQ9	COX5B CYC7 QCR9	SDH2 SDH3 CYB2					PMA1		
Stress response							**Stress response**						
Heat shock (see protein folding too)							**Heat shock (see protein folding too)**						
	HOR7 YRO2 SED5 SAM1	HOR7 YRO2 YGP1	DDR2	MHO1 NNR2 ECM10 SPG1	HSP30 SYM1 GAC1 NTH2	SPG4 RPI1 YGP1					NAT1 SKN7 SSB2 MGA1	SAM1	
Oxidative stress							**Oxidative stress**						
Mitochondria							**Mitochondria**						
PRX1	ALD4 CCP1	CCP1 HIG1	CTT1 MCR1 ALD4 STF2 OM45	CCP1 PRX1							POS5 OGG1		
Glutathione							**Glutathione**						
			GND2	GTT1 GTT2 GPX1 GLO4 GRX2	IDP3 GND2 ECM4						OPT1 SFA1 RNR3 RNR4 IDP1 GSH1 GND1		
Detoxifying reactive oxygen species							**Detoxifying reactive oxygen species**						
CTT1 GAD1	ALD2 GRE2 GRE3	CTT1 GRE3 DAK1 GDP1	GRE2 GRE3 ARA1 ALD2	GAD1 ALD2	GRE2 ALD3	SOD1							
Other							**Other**						
	ALD6 DDR48 TRX2	DDR48 TRX2 TTR1 TRX1 GRX1 SOD2 NCA3 FUN30 FET3	ALD6 ZTA1 DDR48 BDH2 YML131W	TRX2 NCA3 BDH2 YML131W	TSA2 MRX2 FBA1 RNY1 LOT6	RCK1 OXR1					TAH18 GDN1 YHB1 UGA3		

Continuation: Table 18: Comparison of several salt stress and osmotic transcriptional analysis studies with this thesis results. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

Osmotic stress							**Osmotic stress**						
				DOG2 *GPD1* *YML131W*	*SIP18* *MPC3* *GRE1*	*GRE2*					*SKO1*		
Salt stress							**Salt stress**						
											ISC1	*YGK3*	
Ion homeostasis							**Ion homeostasis**						
ENA1 *VHS3*	*ENA1* *ENA5* *VMA7* *VMA5* *VMA6* *VPH1*	*PPZ1* *SIT1*	*ENA1*	*VHS3* *VMA22* *PMA2* *LDH1* *IZH4* *PRM6* *FIT2*	*YKE4* *ZRT1* *ALR2* *ARN2* *RAV2* *AST2*						*SPF1* *PER1* *PCA1* *CCC2* *PMR1* *GGC1* *FTR1*	*CTR1* *FRE1* *FRE4* *ATX2* *CTR3* *TOM5* *RSN1*	*YMR279C* *MRS3* *AFT1* *PMA1* *PKR1*
Cell wall stress							**Cell wall stress**						
				HSP12	*USV1*						*WSC2*	*NCW2*	
Overall stress							**Overall stress**						
				MSC1 *FMP40* *FMP16* *UGX2* *MBR1* *YDR034W-B*	*PFA3* *MAF1* *ALD2* *ALD3* *MRK1* *GPH1*	*SSA3* *MSN4* *SED1* *YRO2* *DCS2*					*CGR1* *MRC1* *CMK2*		
Drug Resistance							**Drug Resistance**						
				PDR3 *PDR8* *CAD1* *PDR11* *YLR046C*	*YPC1* *PAD1* *ARR1* *TAT1* *YNR064C*	*QDR2* *QDR1* *MIG3* *RDS1* *CRG1*					*SNQ2* *SUP45* *FAP1* *KRE33* *RPS14B*	*PBL2* *IMD2* *YLR179C* *SSZ1* *SRO9*	
DNA damage							**DNA damage**						
				HSM3	*PCD1*						*HUG1*		
Starvation							**Starvation**						
				SUT1 *XBP1* *NPP2*	*DSE1* *GSY2* *SNO4*	*SPL2* *SIP2* *YGP1*					*ZPR1* *PUB1* *NRP1*		
Other stress							**Other stress**						
				RTA1 *RIM13*	*TIR1* *TPK2*						*PUG1* *PDR12*		
Protein folding (chaperones and others)							**Protein folding (chaperones and others)**						
HSP12	*HSP10* *HSP42* *HSP78*	*HSP12* *HSP26* *HSP78* *MDJ1* *HSP104* *SSA4* *SSE2* *SSA1* *CPH1* *HSC82*	*HSP12* *HSP26* *HSP42* *HSP104* *SSA4* *SSA3* *SSE2*	*HSP12* *HSP26* *HSP82* *HSP104* *SSA4* *SSA3* *TRX3* *OPI10* *FES1* *TRX2*	*SIS1*						*ZUO1* *HSP10* *NMA111* *SSB1* *KAR2* *SEC63*	Related to assembly of actin and tubulins *CCT8* *CCT4* *CCT5* *CCT3* *CCT7*	
Protein degradation							**Protein degradation**						
Protein degradation (proteosome)							**Protein degradation (proteosome)**						
	PAI3	*PRE1*		*ECM29* *YPS6*	*RPN14* *RPN13*	*VID24* *NNK1*					*CYM1* *MNL1*		
Vacuolar protein degradation							**Vacuolar protein degradation**						
				COG7	*SNA3*	*ATG34*					*CPS1*	*FRD1*	*VID22*
Protein degradation (ubiquitiination)							**Protein degradation (ubiquitiination)**						
		UBC4 *GRR1* *HEL1*		*HEL1* *HRT3* *UBP11*	*UBC8* *UBC12* *SAF1*	*DIA2* *RRI1* *ULP2*					*NPR1*		

Continuation: Table 19: Comparison of several salt stress and osmotic transcriptional analysis studies with this thesis results. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

				UBP8 *PIB1*	*FYV10* *UBP9*	*UBP7*							
Ribosomes, transcription and translation							**Ribosomes, transcription and translation**						
Ribosomal proteins							**Ribosomal proteins**						
							RLP24	*RPS3* *RPS6B* *RPS9B* *RPS10B* *RPS11A* *RPS11B* *RPS15* *RPS18A* *RPS21A* *RPS22A* *RPS26A* *RPS26B* *RPS27B* *RPS28B* *RPS39A* *MRPL8* *MRPL40* *MRP8* *RPL8B* *RPL9A* *RPL12A* *RPL12B* *RPL13A* *RPL21A* *RPL24A* *RPL24B* *RPL28* *RPL35A* *RPL37B* *RPL39* *RPL42B*	*RPS3* *RPS4B* *RPS6A* *RPS7B* *RPS8A* *RPS9A* *RPS11A* *RPS13* *RPS15* *RPS16B* *RPS17B* *RPS18A* *RPS20* *RPS22A* *RPS24B* *RPS25A* *RPS25B* *RPS26A* *RPS26B* *RPS27A* *RPS27B* *RPS31* *MRP8* *MRPS18* *RPL1A* *RPL1B* *RPL9A* *RPL11B* *RPL12A* *RPL13B* *RPL15B* *RPL16B* *RPL19A* *RPL19B* *RPL20A* *RPL21A* *RPL21B* *RPL23A* *RPL25* *RPL32* *RPL33B* *RPL34A* *RPL34B* *RPL35A* *RPL38* *RPL39* *RPL43A*		*RPS0A* *RPS0B* *RPS1A* *RPS1B* *RPS2* *RPS3* *RPS5* *RPS6B* *RPS7B* *RPS8A* *RPS8B* *RPS10A* *RPS11B* *RPS14B* *RPS16B* *RPS17A* *RPS17B* *RPS18B* *RPS19A* *RPS22A* *RPS24A* *RPS25A* *RPS25B* *RPS26B* *RPS27B* *MRPL4* *CLB1* *RPP0* *RPP2A* *RPL1A* *RPL2B* *RPL6B* *RPL7A* *RPL8A* *RPL8B* *RPL9A* *RPL9B* *RPL11A* *RPL12A* *RPL13B* *RPL14B* *RPL15A* *RPL16A* *RPL16B* *RPL17A* *RPL17B* *RPL20A*		*RPL20B* *RPL21A* *RPL21B* *RPL23B* *RPL24A* *RPL24B* *RPL25* *RPL31B* *RPL32* *RPL36A* *RPL43A*
tRNA metabolism							**tRNA metabolism**						
				THG1 *SRL2* *TPT1*				*DED81* *MES1* *KRS1* *DPS1* *FRS2* *GLN4*		*ILS1*	*DED81* *MES1* *KRS1* *GLN4* *ILS1* *TRM1* *SMM1* *PUS7*	*NCL1* *GCD14* *PUS1* *TRM5* *ABP140* *PUS4* *TYW1* *TRL1*	*FRS1* *HTS1* *TYS1* *MSD1* *CDC60* *THS1* *GDC10* *TRM8*

Continuation: Table 20: Comparison of several salt stress and osmotic transcriptional analysis studies with this thesis results. Genes in green have up-regulated expression in S. cerevisiae when compared to S. boulardii in this thesis results and red have down-regulated expression.

									MTO1	WRS1	VAS1
Ribosome biogenesis					**Ribosome biogenesis**						
				PUS5	DHR2	CDC95		NOP1	DBP2	MAK16	RIX1
				GSP2	IPI3			SIK1	ALB1	ERB1	RRP1
				ESF1	DBP2			EGD2	NOP56	URB1	RLI1
					DBP7			SNU13	UTP22	NOP7	DIM1
					ALB1			GAR1	UTP5	RRP17	KRE33
					UTP23			REX4	UTP15	NIP7	NOC4
									UTP9	DBP9	BFR2
									UTP10	RSA3	RRP3
									UTP4	YTM1	RRP5
									NAN1	DBP10	NSR1
									UTP13	NOG2	EMG1
									UTP21	SDA1	ROK1
									DIP2	RRB1	RRP9
									PWP2	DBP3	DBP8
									IMP3	SQT1	NDE1
									IMP4	NOC3	PRP43
									BMS1	ARX1	KAP123
									RCL1	MAK21	SXM1
									NOG1	SPB1	NOP58
									NUG1	NOC2	BTM6
									NOP4	SSF1	SNU13
									UTP18	ARB1	REI1
									RRP12	RSA4	RPP1
									PWP1	MRT4	REX4
									RIX7	PZF1	YVH1
									NOP1	PPT1	
									DRS1	ERP2	
RNA degradation					**RNA degradation**						
				DCS1					CAF16	RRP45	MTR4
									CAF120	DIS3	SRP72
Translation					**Translation**						
				RNY1		HYP2		PAB1	EFT2	TIF5	DPH1
				HEF3		TIF2		TIF35	YEF3	FUN12	RRT2
				PTH1		PRT1		ASC1	SYO1	GCD2	TPA1
				MRPL16		TIF1		EFB1	SHB17	GCD1	SWD2
						TIF11		EFT1	SRP40	STO1	
								EFT2	NOP13	NAM7	
								YEF3	RSM22	CLU1	
								SSB2	RNA1	CAF20	

Overexpressed probiotic functions

Probiotic properties predicted to underlie the function of *S. boulardii* include the expression of: 1) anti-toxin proteins (one of 120 kDa); 2) cell wall and adhesion proteins, such as flocculins; 3) enzymes involved in the biosynthesis and release of polyamines, such as spermidine and spermine; 4) enzymes required for the biosynthesis of SCFA; 5) enzymes leading to lipid degradation in dendritic cells; and 6) enzymes required for galactose and palatinose metabolism, leading to lactase production in the host. As such, the genes related to these biological processes whose expression was found to be different in *S. boulardii* and *S. cerevisiae* were searched for (*Table 21*).

Only one potential anti-toxin gene was found to be overexpressed in *S. boulardii*, when compared to *S. cerevisiae*: *VAS1*. Vas1p is a 120 kDA protein that has been shown to decrease water and sodium secretion in intestinal loops and counteract the increase in cAMP levels in rat intestinal cells done by *Vibrio cholerae*'s toxin, leading to toxicity inhibition.

Key genes required for polyamine biosynthesis were also found to be up-regulated in *S. boulardii*, when compared to *S. cerevisiae*. As mentioned in the Introduction, many digestive enzymes (sucrase-isomaltase, maltase-glucoamylase, lactase-phlorizin hydrolase, alanine aminopeptidase and alkaline phosphatase) and nutrient transporters (sodium-glucose transport proteins) activity may be induced by polyamines secreted by *S. boulardii*. Secreted polyamines also promote RNA binding and stabilization and, hence, growth and differentiation proteins (lactase, maltase, sucrase, among others) synthesis. These molecules are also able to defend lipids from oxidation and boost SCFA activity. The enzymes will probably then participate in the GRB2-SHC-CrkII-Ras-GAP-Raf-ERK1,2 pathway and PI3K pathway. Polyamines may also affect kinase activities and external signals, furthering modulation these two pathways. All of these polyamines functions lead to a general polyamine-triggered metabolic activation in order to regenerate brush border damaged areas quickly. [1,4,66,67] Released spermine and spermidine are then absorbed and lead to brush border membrane.[1] Thus, it is very significant that, in the ILM medium used herein, the expression of *SPE2* and *SPE3*, encoding the enzymes that catalyse the steps for spermidine biosynthesis is higher in *S. boulardii*. In terms of the excretion of polyamines the results are less clear, as one polyamine exporter encoding gene, *TPO2*, is overexpressed, while two others, *TPO1* and *TPO4*, are under-expressed in *S. boulardii*, when compared to *S. cerevisiae*.

S. boulardii produces SCFAs, such as acetic acid and propionic acid, acetic acid in itself making up 50% of total SCFAs in the colon. Many diseases cause imbalance of SCFA concentrations in the colon and *S. boulardii* has been shown to correct these imbalances. It has also been shown that *S. boulardii* produces moderate amounts of acetic acid, whereas *S. cerevisiae* produces insignificant amounts. Furthermore, *S. boulardii* strains with causative SNP mutations in *SDH1* and *WHI2* produces high amounts of acetic acid. It has also been shown that acetic acid has significant antimicrobial properties. In this context, although no changes in *SDH1* or *WHI2* gene expression could be detected, the expression of other genes encoding acetic acid generating enzymes, namely *ALD5*, *MET17*, SFC1, *ACS2*, was found to be up-regulated in *S. boulardii* when compared with *S. cerevisiae*, while two other, *ALD2* and *ALD3*, were down-regulated. Down-regulation of *ALD2* and *ALD3* is not very significant, since these two genes encode aldehyde dehydrogenase that may participate unspecifically in acetate

synthesis. *ALD5,* however, encodes a acetaldehyde dehydrogenase specifically involved in acetate synthesis, while Sfc1p is a mitochondrial succinate-fumarate transporter required for acetate utilization. Met17p is an O-acetyl homoserine-O-acetyl serine sulfhydrylase required for methionine and cysteine biosynthesis that releases acetate in the process. Even though Acs2p, an acetyl-coA synthetase, degrades acetate, it is also involved in the production of propionate, another SCFA. It is thus reasonable to hypothesize that the overexpression of these SFCA biosynthesis related genes may contribute to the probiotic phenotype of *S. boulardii*.

Galactose metabolism related genes *PGM1*, *CYC8* and *TUP1* were up-regulated in *S. boulardii* when compared with *S. cerevisiae*. Not only does *S. boulardii* induce the enzymatic activities of lactase-phlorizin hydrolase, α-glucosidases, alkaline phosphatases and aminopeptidases, but also increases D-glucose intestinal absorption, one of the products of lactose degradation. Production of lactase by the host and its overexpression by *S. boulardii* leads to lactose degradation, which can help in lactose intolerance. [16,19]

One palatinose metabolism gene was up-regulated in *S. boulardii* when compared with *S. cerevisiae*. This overexpression may contribute to *S. boulardii* probiotic activity as its role appears to require sucrose isomaltase expression, together with the stimulation of sucrose isomaltase, as a digestive enzyme, in the host (trophic effect). [16]

Table 21: Genes associated with the main probiotic properties of S. boulardii. Genes in green and red are down and up-regulated, respectively, in S. boulardii when compared with S. cerevisiae.

63-kDa Phosphatase	*PHO8*	*SNM1*	*YDR476C*	*CWC21*	*JIP4*	*KRE2*	*RIB3*		
	PRP3	*PEX29*	*SNF1*	*VPS72*	*DIG2*	*VP60*	*PAC11*		
54-kDa serine protease	*PCR1*	*YSP3*	*YBR139W*	*RRT12*					
120 kDa protein	*KIN1*	*PIK1*	*VAS1*	*RGC1*	*TFC4*	*PUF*	*ENA5*	*SEG2*	
	MAD1	*NMD5*	*KAP12*	*NUP120*	*JSN1*	*MSH3*	*KCS1*		
Cell wall proteins, adhesion proteins and flocculins	*AGA2*	*FIG2*	*CWH41*	*KTR1*	*CRH1*	*EXG1*	*SPR1*	*SPI1*	*SCW11*
	BGL2	*FIT1*	*CWP2*	*LAS21*	*CRR1*	*EXG2*	*SRL1*	*SMK1*	*EFG1*
	CCW12	*FIT2*	*DCW1*	*MNT2*	*HKR1*	*ROT2*	*SUN4*	*SKN1*	
	CIS3	*FKS3*	*DFG5*	*PIR3*	*KNH1*	*KRE6*	*UTR2*	*SHE10*	
	CKA2	*GSC2*	*DSE2*	*ST1*	*YPS3*	*SCW10*	*YPS1*	*SCW4*	
	FIG2	*FLO10*	*AGA1/SAG1*	*FLO11*	*FLO5*	*FLO8*	*FLO1*	*FLO9*	
Polyamines, spermidine and spermine	*SPE2*	*CAR2*	*CAR1*	*PRO1*	*SPE3*	*PUT2*	*PRO3*	*PRO2*	
	TPO1	*QDR3*	*TPO4*	*AGP2*	*SAM3*	*TPO2*	*PUT1*		
SCFA (acetic acid and propionic acid)	*ALD2*	*ALD4*	*ALD6*	*TDA9*	*ACS1*	*SFC1*	*STR2*		
	ALD3	*ALD5*	*ACH1*	*ZMS1*	*ACS2*	*MET17*			
Lipid degradation in dendritic cells	*TGL2*	*TGL5*	*TGL4*	*YJU3*	*TGL3*	*AYR1*	*YDC1*	*TGL1*	*YPC1*
Galactose metabolism	*GAL1*	*GAL7*	*GAL10*	*MIG1*	*PGM1*	*CYC8*	*GAL2*	*GAL4*	*GAL80*
	PGM2	*GAL3*	*TUP1*						
Palatinose metabolism	*IMP1*	*IMA1*	*IMP5*	*IMA4*	*IMP2*	*IMA2*	*IMA3*	*IMA5*	
Lactase production (associated with IgA)	*MIG1*	*GAL1*	*GAL10*	*PGM2*	*GAL7*	*GAL80*			
	PGM1	*CYC8*	*GAL4*	*GAL3*	*GAL2*	*TUP1*			

Among the cell wall and adhesion related genes that have been predicted to play an eventual role in *S. boulardii* probiotic effect, only *HKR1* and *YPS1* genes display increased expression levels in

S. boulardii, when compared to *S. cerevisiae*. Yps1p is a GPI-anchored plasma membrane attached aspartic protease with a role in cell wall integrity and adhesion and Hrk1p is a mucin family member that functions as an osmosensor in the HOG pathway, with a predicted role as an adhesin like protein. The over-expression of both these genes suggests *S. boulardii* may display increased adhesiveness then *S. cerevisiae*. On the contrary, however, the flocculin encoding genes *FLO5* and *FLO9* were found to have higher expression levels in *S. cerevisiae*, which suggests the opposite.

S. boulardii has been proposed to be capable of adhering to intestinal mucus membrane and avoiding adhesion of other pathogens flowing by to the intestine.[15] Indeed, it has been shown that *S. boulardii* is able to colonize the intestine of gnotobiotic mice after single administration. However, other studies contradict this notion and say that *S. boulardii* does not strongly adhere to intestinal epithelial cells and is quickly removed from the gastrointestinal system in healthy individuals. This may mean that although *S. boulardii* can colonize the intestine, competition with intestinal microbiome limits it, unless the microbiome is depleted, such as under antibiotic therapy.[21]

In order to evaluate if *S. boulardii* is indeed able to adhere to epithelial intestinal cells, and if it is more able to do so than the non-probiotic yeast S. cerevisiae, adhesion assays were conducted for both species, against FHs 74 Int small intestine cell line (ATCC® CCL-241™). *S. boulardii* not only was able to successfully adhere to epithelial intestinal cells, but it did so exceedingly better than *S. cerevisiae*. From three replicate essays, *S. boulardii* had an average of 58% of adhesion, whereas *S. cerevisiae* had an average of 30% of adhesion, almost half of *S. boulardii*'s adhesion (*Figure 10*).

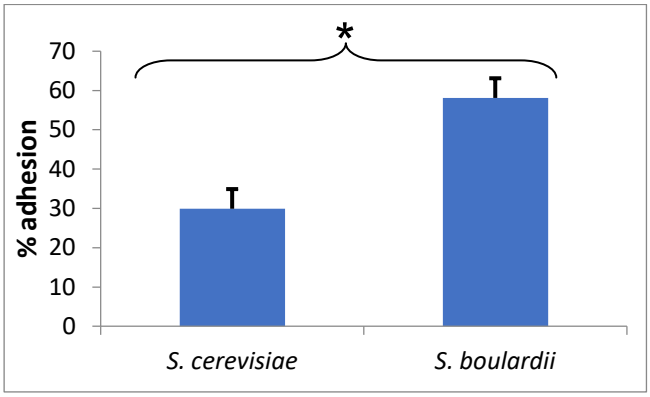


Figure 10: Adhesion percentage of S. boulardii and S. cerevisiae to FHs 74 Int small intestine cell line. Statistical analysis of the results was performed using analysis of variance and differences were considered significant for $p < 0.05$.

Altogether, these results support the notion that *S. boulardii* has a stronger capacity to adhere to intestinal epithelial cells than *S. cerevisiae*, a phenotype that may underlie its increased probiotic capacity. It would be interesting to assess if this phenotypic difference relies on the increased expression of *HKR1* and *YPS1* genes, observed in planktonic cultivation in intestinal tract like medium, or eventually of other adhesion related genes, whose expression can vary in biofilm formation assays or in the presence of intestinal epithelial cells.

ProBioYeastract platform's new computational functionality

Yeastract+ (Yeast Search for Transcriptional Regulators And Consensus Tracking +) is a comparative genomics platform that allows transcriptional regulatory networks analysis for certain yeast species. This platform serves as a doorway to four different interconnected databases: Yeastract, PathoYeastract, N.C. Yeastract and ProBioYeastract. Yeastract database deals only with model yeast *S. cerevisiae*. PathoYeastract focus on pathogenic yeasts, currently including *Candida glabrata*, *albicans*, *parapsilosis* and *tropicalis*, which are responsible for 90% of all detected candidiasis. N.C. Yeastract deals with non conventional yeasts that are pertinent in biotechnology, currently this includes *Z. bailii*, *K. marxianus*, *Y. lipolytica*, *K. phaffii* and *K. lactis*. ProBioYeastract focus on probiotic species, currently including *S. boulardii* biocodex and unique28 strains. Each database is a curated repository of published transcriptional associations used to predict and visualize gene and genomic regulation through comparative genomics, considering orthologous regulatory associations from other yeast. With this tool it is possible both to identify documented or potential transcription regulators of a gene and to compare DNA motifs and transcription factors binding sites.[100–105]

In this thesis, a new functionality was implemented for the ProBioYeastract platform. This new tool finds transcription factors that specifically regulate the inputted genes. This is done by comparison of transcription factor's binding sites (TFBS) within the promoter's sequences and computation of which ones match. The species from which the TFBS are considered are selected by the user. At the moment, there is no such information available for *S. boulardii* in the literature, so only *S. cerevisiae* data can be used. Promoter's sequence is both from *S. cerevisiae* and *S. boulardii*. The matches are then separated in three groups: when they are found in both *S. cerevisiae* and *S. boulardii* or when they are found in only one of them. With this information a table is constructed with each group as a column and each transcription factor as a line. In each cell are the input genes or their homologs in the remaining species, regulated by that line's transcription factor.

It is now possible to predict which input genes a certain transcription factor regulates. However, we do not know if that transcription factor regulates only a small group of genes (as in, mostly the input genes and few others) or if it regulates a huge amount of genes besides the input genes. In the first case, the transcription factor would be highly interesting, since it would suggest a specific regulation, while in the latter case a general non-specific regulation is suggested. To analyze how specific the regulation is, a p-value is calculated for each pair of transcription factor and respective regulated genes through the hypergeometric distribution.[106] The hypergeometric distribution takes as input the size of the sample, the size of the population, number of successes in the sample and number of successes in the population. In this case, the population size is the number of genes the species has, while the sample size is the number of genes the user has inputted. The number of successes in a given species population is the number of genes whose promoter contains at least one binding site of a given transcription factor, whereas the number of successes in the sample is the number of inputted genes whose promoter of that species contains at least one binding site of a given transcription factor. This p-value is then displayed in the table for each transcription factor – target

genes cluster; in other words, there is one p-value for each line and column that is independent of other p-values in other lines.

However, some p-values can be very similar to each other and it would be difficult to translate that value meaningfully. In order to create a bigger scale and separate the p-values from each other, the Bonferroni correction was used.[107] This correction relates p-values from different lines by taking into account the size of the table. This is done by dividing the p-value by the number of lines (or transcription factors).

Hence, we finally obtain a table with each species (or intersection of them) as a column, each transcription factor as a line and each cell with the input genes regulated by that line's transcription factor for that column's species or the respective corrected p-value. A pseudocode version of this new functionality's code can be found below (*Figure 11*). An example form and table can be seen in *Figure 13* and *Figure 12*.

Figure 11: Pseudocode version of this new functionality's code.

User input:
    UserGenes <- *S. boulardii* biocodex's or unique28's orfs/genes
    TFBSSpecies <- species of transcription factors binding sites
UserSpecies <- GetSpecies(**UserGenes**)
UserHomologousSpecies <- GetHomologousSpecies(**userSpecies**)
TFandTFBS <- GetTFBS(**TFBSSpecies**)
For each SP in (**UserSpecies** U **UserHomologousSpecies**)
    PromotersPerSpecies <- GetPromoters(SP)
Matches <- GetMatches(TFand**TFBS, PromotersPerSpecies)**
Intersect <- Get**RegulatedGenes**PresentIn**AllSpecies(Matches)**
UniquePerSpecies <- Get**RegulatedGenes**PresentOnlyIn**OneSpecies(Matches)**
For each TF in **TF**andTFBS
    Foreach SP in **UserSpecies** U **UserHomologousSpecies**
    P-value <- **CalculateP-Value** (**TF, SP, Matches**)
    CorrectedP-Value <- Use**BonferroniCorrection**OnP-Value (**P-value**)

Howsoever, it should be noted that getMatchesBetweenTFBSandPromoters is a very heavy function. In total, the database has 533 transcription factors with documented regulation, but only 188 transcription factors have documented binding sites. From those, only 124 transcription factors belong to *S. cerevisiae*. However, each transcription factor can have many binding sites and, in the case of *S. cerevisiae*, there are 401 binding sites for all of the 124 transcription factors. Since there isn't information about *S. boulardii*'s transcription factors' binding sites, it is these 401 binding sites that can be compared with the promoter's sequences of each species (6823, 5482 and 5493 promoter sequences for *S. cerevisiae*, *S. boulardii* biocodex and *S. boulardii* unique 28, respectively) (*Table 22*). This means that to compute all matches for the population (genes the species has), the

getMatchesBetweenTFBSandPromoters function will have to compare TFBS and promoter's sequences $401\text{x}(6823 + 5482 + 5493) = 7\,136\,998$ times. If we consider that each comparison takes 0.01 seconds, all matches would take 71 369.98 seconds (or about twenty hours) to run. This waiting time is not acceptable for a web page to load and, in order to avoid this, all matches between transcription factors and database's set of genes, for all species, were pre-computed and a table was added to the database with information about transcription factors and how many promoters' they regulate (how many matches they are associated to) for each species. This table does not however contain information about which promoters or genes are regulated or which binding site the transcription factor recognizes, only the total number of matches. Hence, there is no need to perform the 7 136 998 matches for all population in order to calculate the p-value each time someone uses the new functionality, since it is possible to just take the result from the new database table. Nonetheless, matches for the input genes still have to be calculated each time someone uses the new functionality, since each time different genes are inputted. Considering that differential gene expression analysis through total RNA-sequencing can lead to thousands of differently expressed genes (1100 in the transcriptome analysis conducted herein), this would still mean about 1100 promoters and hence 441 100 comparisons to make. If we consider that each comparison takes 0.01 seconds, all matches would take 4411 seconds (or about one hour and fifteen minutes) to run. To speed up the use of this function, the matches for each gene would have to be calculated and stored individually, in order to be easily accessible in the database.

Table 22: Number of transcription sites, TFBS and promoters per species present in the database.

	Transcription factors	**Transcription factors' binding site**	**Promoters**
S. cerevisiae	124	401	6823
***S. boulardii* biocodex**	-	-	5482
***S. boulardii* unique 28**	-	-	5493

As an example, down-regulated genes in *S. boulardii* when compared with *S.* cerevisiae from osmotic stress and ion metabolism were used as input to find out relevant differences in regulation by transcription factors. The top part of the table obtained can be seen in *Figure 13.*

Many transcription factors either regulated all genes in both strains and no genes uniquely in any of the strains (*Figure 15*) or regulated most of genes in both strains with few genes uniquely in any of the strains (*Figure 15*). Several transcription factors did not regulate any of the inputted genes or regulated very few of them very unspecifically (*Figure 16*). Some transcription factors regulated few genes in both strains and a good amount in only one of the strains, but this last group of genes is not specifically regulated by said transcription factor. For example, transcription factors Ace2p regulates no genes in both strains, 2 genes only in *S. cerevisiae* and 4 genes only in *S. boulardii*. However, its p-value is very high, indicating that Ace2p regulates way more genes than only those represented in the table and is not specific for those at all (*Figure 17*). The ideal would be to find a transcription factor with very low specificity for genes regulated in *S. cerevisiae* and very high specificity for genes regulated only in *S. boulardii*. Even better if there are few or no genes regulated in both strains. However, such transcription factor was not clearly found.

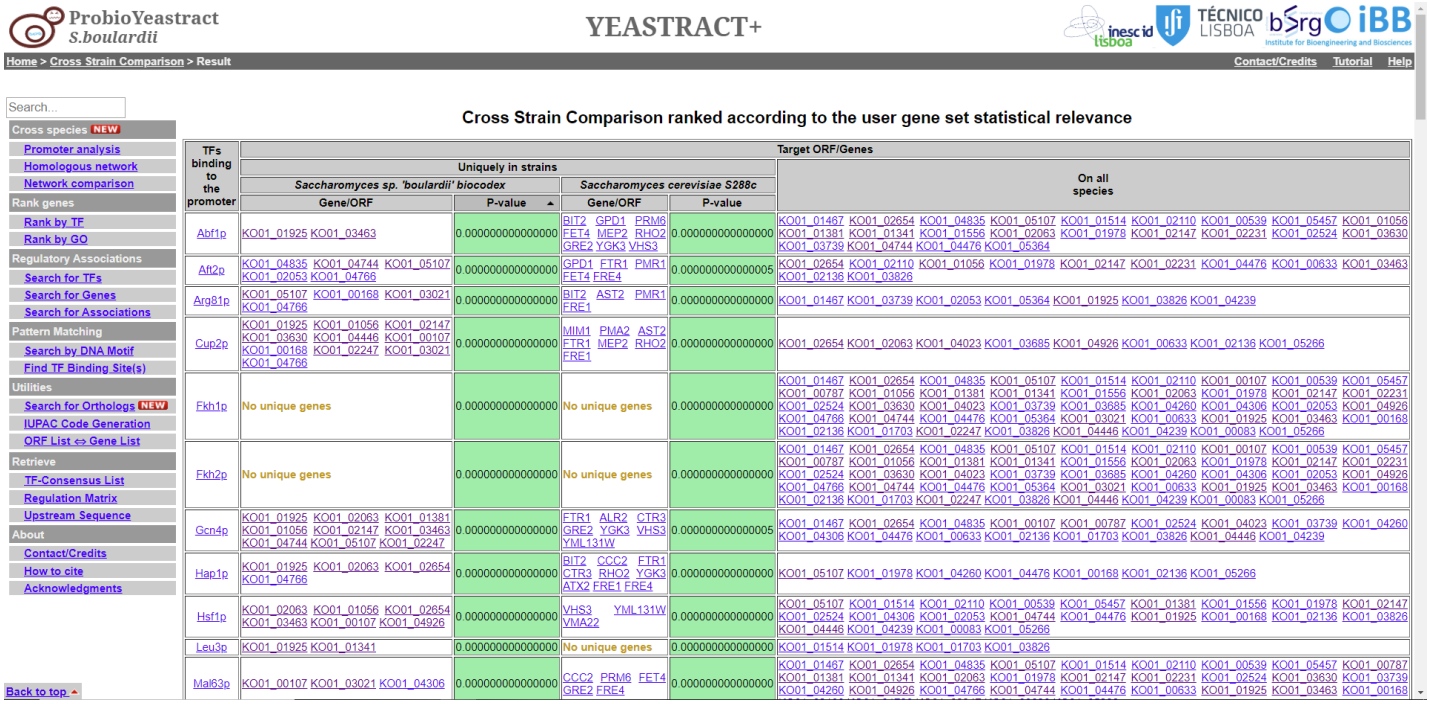


Figure 12: Cross Strain Comparison ranked according to the user gene set statistical relevance for down-regulated genes in S. boulardii *when compared with* S. *cerevisiae from osmotic and salt stress*

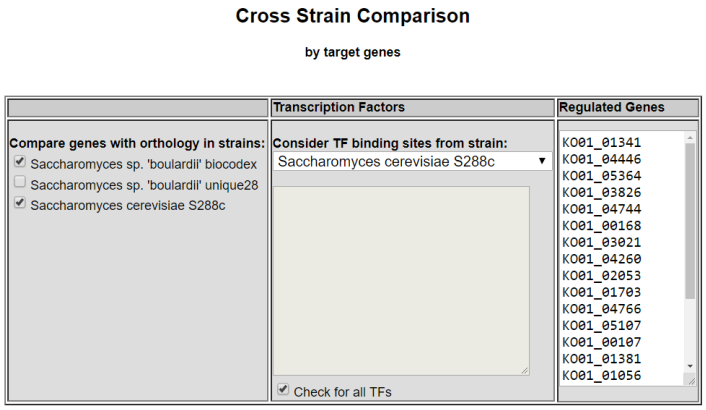


Figure 13: Example Form

Fkh1p	No unique genes	0.000000000000000	No unique genes	0.000000000000000	KO01_05107 KO01_00107 KO01_00787 KO01_01056 KO01_01381 KO01_01341 KO01_02231 KO01_03630 KO01_04260 KO01_02053 KO01_04766 KO01_04744 KO01_05364 KO01_03021 KO01_00168 KO01_02136 KO01_01703 KO01_02247 KO01_03826 KO01_04446
Fkh2p	No unique genes	0.000000000000000	No unique genes	0.000000000000000	KO01_05107 KO01_00107 KO01_00787 KO01_01056 KO01_01381 KO01_01341 KO01_02231 KO01_03630 KO01_04260 KO01_02053 KO01_04766 KO01_04744 KO01_05364 KO01_03021 KO01_00168 KO01_02136 KO01_01703 KO01_02247 KO01_03826 KO01_04446

Figure 14: Example of transcription factors that regulated all genes in both strains and no genes uniquely in any of the strains.

Rtg1p	No unique genes	0.000135293550736	GRE2	0.000000000000000	KO01_05107 KO01_00107 KO01_00787 KO01_01056 KO01_01381 KO01_01341 KO01_02231 KO01_03630 KO01_04260 KO01_04766 KO01_04744 KO01_05364 KO01_03021 KO01_00168 KO01_02136 KO01_01703 KO01_02247 KO01_03826 KO01_04446
Rtg3p	No unique genes	0.000135293550736	GRE2	0.000000000000000	KO01_05107 KO01_00107 KO01_00787 KO01_01056 KO01_01381 KO01_01341 KO01_02231 KO01_03630 KO01_04260 KO01_04766 KO01_04744 KO01_05364 KO01_03021 KO01_00168 KO01_02136 KO01_01703 KO01_02247 KO01_03826 KO01_04446

Figure 15: Example of transcription factors that regulated most of genes in both strains with few genes uniquely in any of the strains.

Arg80p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Cat8p	No matches	1.000000000000000	PMA2 FTR1	0.000512805835555	No common genes
Dal80p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Dal82p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Fzf1p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Hcm1p	No matches	1.000000000000000	CTR3	0.231959811262057	No common genes
Mac1p	No matches	1.000000000000000	CCC2 FTR1	0.005949099998427	No common genes
Met4p	No matches	1.000000000000000	GPD1 VHS3	0.162476626184427	No common genes
Rap1p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Reb1p	No matches	1.000000000000000	FTR1	0.000008163878079	No common genes
Rme1p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Rpn4p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Sip4p	No matches	1.000000000000000	PMA2 FTR1	0.000512805835555	No common genes
Smp1p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Uga3p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Ume6p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Cad1p	No matches	1.000000000000000	AST2	0.931783456048740	No common genes
Cin5p	No matches	1.000000000000000	AST2	0.931783456048740	No common genes
Arr1p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Flo8p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Mss11p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Rfx1p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Kar4p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Ixr1p	No matches	1.000000000000000	No matches	1.000000000000000	No common genes
Yap5p	No matches	1.000000000000000	AST2	0.931783456048740	No common genes
Tye7p	No matches	1.000000000000000	GPD1 VHS3	0.162476626184427	No common genes
YHR177w	No matches	1.000000000000000	No matches	1.000000000000000	No common genes

Figure 16: Example of transcription factors that did not regulate any of the inputted genes or regulated very few of them are very unspecifically

Ace2p	KO01_03630 KO01_04446 KO01_00107 KO01_00787	0.864164710163910	FTR1 MEP2	0.959615793457813	No common genes
Swi5p	KO01_03630 KO01_04446 KO01_00107 KO01_00787	0.864164710163910	FTR1 MEP2	0.959615793457813	No common genes

Figure 17: Example of transcription factors that regulated genes uniquely in one strain but very unspecifically.

The most relevant results found were for transcription factors Yap3p and Gcn4p (*Figure 18* and *Figure 19*, respectively). Yap3p regulates only one gene (*AST2*) in both strains, 4 uniquely in *S. cerevisiae* (*GPD1*, *GRE2*, *VHS3* and *YML131W*) and 4 uniquely in *S. boulardii* (*CCC2*, *ATX2*, *PMA2* and *VMA22*). Gpd1p is an enzyme involved in glycerol synthesis that is essential for growth under osmotic stress. Its expression is regulated by high-osmolarity glycerol response pathway. Vhs3p is the negative regulatory subunit of protein phosphatase 1 (Ppz1p) involved in cellular metal ion homeostasis. Ppz1p is involved in regulation of potassium transport, which affects osmotic stability, cell cycle progression, and halotolerance. *GRE2* is a stress induced gene (osmotic, ionic, oxidative, heat shock and heavy metals) regulated by the HOG pathway. *YML131W* gene is induced by stresses including osmotic shock, DNA damaging agents, and other chemicals. Ccc2p is a Cu^{+2}-transporting P-type ATPase required for export of copper. Atx2p is involved in manganese homeostasis. Pma2p is a plasma membrane H^{+}-ATPase involved in pumping protons out of the cell and, hence, regulating cytoplasmic pH and plasma membrane potential. Vma22p is a peripheral membrane protein required for vacuolar H^{+}-ATPase (V-ATPase) function. All of these genes are down-regulated in *S. boulardii* when compared with *S. cerevisiae*. As we can see Yap3p not only regulates different genes in *S. cerevisiae* and *S. boulardii*, but up-regulates them in *S. cerevisiae*.

Yap3p is an AP-1 type transcription factor that belongs to the YAP protein family. This protein family is composed by stress response and metabolism control pathways transcription activators with different yet identical DNA binding specificities. Normally they activate transcription from promoters containing a Yap recognition element. Although its regulatory targets aren't well known, Yap3p seems to be involved in hydroquinone cellular response and aminotriazole and benzenic compounds stress

response. Overexpression of Yap3p induces *PDR5* transcription and increases 4-nitroquinoline-N-oxide and rapamycin resistance. Pdr5p is a multidrug resistance ABC transporter that increases tolerance to fluconazole and cycloheximide fungicides.[108–111]

Null mutants are viable, but become sensitive to endoplasmic reticulum stress created due to promotion of unfolded protein response by tunicamycin, to arsenic or monomethylarsonous treatment and to hydroquinone. They also have abnormal growth and vacuolar morphology, diminished hyperosmotic and alkaline pH stress tolerance. However, these mutants are more resistant to acid and DNA-damaging agents. A transcriptional study showed that Yap3p is up-regulated in response to cumene hydroperoxide, an oxidative stress inducer. [108–111]

Hence, Yap3p seems to be involved in multidrug resistance and unfolded protein response and possibly also in oxidative, hyperosmotic and alkaline pH stress tolerance. Even though this transcription factor is said to be involved in hyperosmotic stress, its involvement is not yet well known. Since this transcription factor was found to be relevant according to the bioinformatic tool used, it would be interesting to further analyze its behavior in laboratorial experiments under hyperosmotic conditions [108–111]

It should be noted that Yap3p is down-regulated in *S. boulardii* when compared to *S. cerevisiae*. Hence it makes sense that the genes regulated in both species are up-regulated in *S. cerevisiae*. As it was seen, *GPD1*, *GRE2*, *VHS3* and *YML131W* genes are regulated by Yap3p uniquely in *S. cerevisiae*. This means that Yap3p might have lost these genes as targets in *S. boulardii*. This does not contradict the transcriptomics results, since Yap3p is a transcription factor that activates genes expression and these genes were shown to be down-regulated in *S. boulardii*. Altogether, this analysis suggests that the predicted loss of Yap3p targets in *S. boulardii*, when compared to *S. cerevisiae*, may explain its decreased osmotic stress resistance. It would, thus, be interesting to evaluate if by increasing the expression of these lost target genes in *S. boulardii*, it would be possible to increase its tolerance to osmotic stress and promote an even better probiotic performance of this species.

Gcn4p is predicted to regulate 7 genes (*BIT2*, *GPD1*, *MEP2*, *ZRT1*, *MPC3*, *SIP18* and *FRE4*) in both strains, 5 uniquely in *S. cerevisiae* (*FTR1*, *CTR3*, *GRE2*, *VHS3* and *YML131W*) and 5 uniquely in *S. boulardii* (*AST2*, *CCC2*, *ATX2*, *PMA2* and *VMA22*). Ftr1p and Ctr3p are high affinity iron and copper permease, respectively, involved in the transport of iron and copper, respectively, across the plasma membrane. All of the genes uniquely regulated in *S. cerevisiae* are up-regulated in *S. cerevisiae* when compared to *S. boulardii*. Ast2p is involved in targeting of plasma membrane H^+-ATPase (Pma1p) to the plasma membrane. All of the genes uniquely regulated in *S. boulardii* are down-regulated in *S. boulardii* when compared with *S. cerevisiae*. As we can see Gcn4p not only regulates different genes in *S. cerevisiae* and *S. boulardii*, but up-regulates them in *S. cerevisiae*, whereas it down-regulates them in *S. boulardii*. It is interesting to note that both Yap3p and Gcn4p regulate similar genes uniquely in *S. boulardii*.

Gcn4p is an amino acid synthesis' genes transcriptional activator, especially during amino acid starvation. This transcription factor regulates more than 30 genes required for amino acid or purine synthesis, but also genes involved in autophagy, multiple stress responses, glycogen homeostasis, and organelle biosynthesis. It has been shown that this transcription factor is induced in other conditions besides amino acid starvation: purine starvation, glucose limitation, growth on ethanol, high salinity medium and treatment with methyl methanesulfonate or rapamycin.[112–114]

Indeed, Gcn4p is crucial for high salt stress survival due to Hal1p transcriptional activation. Hal1p is crucial for maintaining Na^+ and K^+ ion homeostasis and has a CRE in its promoter. This protein is induced both during osmotic and salt stress. A *GCN4* mutant shows sensitivity to elevated potassium or sodium concentrations in the medium. Both Gcn4p and Sko1p compete antagonistically to bind to the CRE site. While Sko1p acts as a transcriptional repressor under normal growth conditions (causing down-regulation of *HAL1*), Gcn4p acts as a transcriptional activator during hyperosmotic stress (causing up-regulation of *HAL1*). However, only modest *GCN4* induction aids in salt stress survival, since exaggerated overexpression of these gene impedes growth. HOG pathway is responsible for stopping Sko1p repression. This repression is however not enough for increased *HAL1* expression, since an activator is necessary. During salt stress, Gcn4p represses the expression of translation factors and also leads to reduced protein synthesis. [112–114]

It would be interesting to know if *AST2*, *CCC2*, *ATX2*, *PMA2* and *VMA22* gene expression is regulated at CRE motifs. In case they are, it may be possible that Sko1p was still repressing these genes expression in *S. boulardii* or that Gcn4p was not able to bind properly to the CRE site. The same could happen for genes *FTR1*, *CTR3*, *GRE2*, *VHS3* and *YML131W.* In case they are regulated at CRE motifs, it may be possible that Sko1p was no longer repressing these genes expression in *S. cerevisiae* and that Gcn4p was able to bind properly to the CRE site. This would make sense since Sko1p transcription factor is up-regulated in *S. boulardii* when compared to *S. cerevisiae* and, hence, more available to repress gene expression in *S. boulardii*. Gcn4p is not differently expressed in *S. boulardii* and *S. cerevisiae*. This is interesting since it might explain why *S. cerevisiae* has an apparently higher ability to respond to osmotic stress than *S. boulardii* to survive in ILM medium.

It would be highly interesting to analyze these two transcriptions factors in the laboratory under hyperosmotic and/or salt stress and compare the results with the obtained with this bioinformatic tool. It is also interesting to note that *YAP3* and *SKO1* are up and down-regulated, respectively, in *S. cerevisiae* when compared with *S. boulardii*.´

As it was seen, *FTR1*, *CTR3*, *GRE2*, *VHS3* and *YML131W* genes are regulated by Gcn4p uniquely in *S. cerevisiae*. This means that Gcn4p lost these genes as targets in *S. boulardii*. Altogether, this analysis suggests that the predicted loss of Gcn4p targets in *S. boulardii*, when compared to *S. cerevisiae*, may explain its decreased osmotic stress resistance. It would, thus, be interesting to evaluate if by increasing the expression of these lost target genes in *S. boulardii*, it would be possible to increase its tolerance to osmotic stress and promote an even better probiotic performance of this species.

Yap3p	KO01_01056 KO01_04744 KO01_05107 KO01_02247	0.000000000000029	GPD1 GRE2 VHS3 YML131W	0.000000000014650	KO01_01381

Figure 18: Example of transcription factor Yap3p.

Gcn4p	KO01_01381 KO01_01056 KO01_04744 KO01_05107 KO01_02247	0.000000000648700	FTR1 CTR3 GRE2 VHS3 YML131W	0.000000004579826	KO01_00107 KO01_00787 KO01_04260 KO01_02136 KO01_01703 KO01_03826 KO01_04446

Figure 19: Example of transcription factor Gcn4p.

Conclusions and Perspectives

Gut dysbiosis refers to the microbiome's quantitative and qualitative composition alterations. Probiotics are a promising treatment or adjuvant to diseases that lead to gut dysbiosis, such as pediatric diarrhea, antibiotic-associated diarrhea, *H. pylori* infection, inflammatory bowel diseases, irritable colon syndrome, acute diarrhea in adults and *Clostridium difficile* infections and *S.boulardii* has shown very promising probiotic properties in most of these disesases without posing a safety risk. [1, 2,4] Probiotics must be able to endure in adverse conditions. The main obstacles in the small intestine are the high concentrations of bile salts, pancreatic enzymes, hydrolytic enzymes, pancreatin, organic acids, the integrity of the epithelial and brush border, the immune defense and the native microbiota. Although *S. cerevisiae* and *S. boulardii* share 95% homology, only *S. boulardii* is considered a probiotic, which is puzzling. [11, 8]

In this thesis, we have grown *S. boulardii* and *S. cerevisiae* in an intestinal like medium (ILM) based on SIEM medium and performed a transcriptional analysis in order to try and understand the different survival and probiotic characteristics of these two very similar strains.

When growing both yeasts in ILM, it was concluded that *S. boulardii* was sensitive to salt stress, especially to bile salts, when compared to *S. cerevisiae*. Furthermore, *S. boulardii* seemed to need a higher period of adaptation to ILM medium than *S. cerevisiae*. However, both strains were eventually able to resume exponential growth, reaching similar maximum final biomass levels.

Transcriptional expression of both strains was compared and, even though, *S. boulardii* and *S. cerevisiae* share 95% homology, their global transcriptional expression differed greatly when grown in ILM medium. Genes whose expression was up-regulated in *S. cerevisiae* when compared to *S. boulardii* appear to suggest that *S. cerevisiae* was feeling a lot more stress than *S. boulardii*, especially in terms of heat shock and oxidative stress. In the case of osmotic stress, the obtained results suggest that *S. cerevisiae* appeared to be more responsive to this stress felt ILM medium. Since apparently *S. cerevisiae* was found to grow better than *S. boulardii* in the presence of high salt concentrations, it is reasonable to hypothesize that it is the apparently higher ability to respond to osmotic stress that enables *S. cerevisiae* to survive in such conditions, when *S. boulardii* cannot. It was also suggested that glycerophospholipids and ergosterol biosynthesis up-regulation and fatty acid degradation down-regulation in *S. boulardii* when compared to *S. cerevisiae* might be deployed to fight membrane stress due to lipase and bile acid effects, suggesting that *S. boulardii* is more sensitive to the effect of these stress agents than *S. cerevisiae*. This observation is consistent with the higher sensitivity exhibited by *S. boulardii* to bile salt concentration.

Potential anti-toxin *VAS1* gene was found to be overexpressed in *S. boulardii*, when compared to *S. cerevisiae*. Vas1p is a 120 kDA protein that has been shown to decrease water and sodium secretion in intestinal loops and counteract the increase in cAMP levels in rat intestinal cells done by *Vibrio cholerae*'s toxin, leading to toxicity inhibition.

The expression of *SPE2* and *SPE3*, encoding the enzymes that catalyse the steps for spermidine biosynthesis is higher in *S. boulardii*. The up-regulation of spermidine synthesis genes is

important, since many digestive enzymes and nutrient transporters activity may be induced by polyamines secreted by *S. boulardii*. Polyamines are also able to defend lipids from oxidation and boost SCFA activity. The sum of all of polyamines functions leads to a general polyamine-triggered metabolic activation in order to regenerate brush border damaged areas quickly. [1,4,66,67]

ALD5, MET17 and *SFC1* were up-regulated in *S. boulardii* and this gene encodes an acetaldehyde dehydrogenase specifically involved in acetate synthesis, while Sfc1p is a mitochondrial succinate-fumarate transporter required for acetate utilization. Met17p is an O-acetyl homoserine-O-acetyl serine sulfhydrylase required for methionine and cysteine biosynthesis that releases acetate in the process. Many diseases cause imbalance of SCFA concentrations in the colon and *S. boulardii* has been shown to correct this imbalances. It has also been shown that *S. boulardii* produces moderate amounts of acetic acid, whereas *S. cerevisiae* produces insignificant amounts. Furthermore, *S. boulardii* strains with causative SNP mutatins in *SDH1* and *WHI2* produces huge amounts of acetic acid. It has also been shown that acetic acid has significant antimicrobial properties.

Cell wall and adhesion related genes *HKR1* and *YPS1* genes displayed increased expression levels in *S. boulardii*, when compared to *S. cerevisiae*. The over-expression of both these genes suggests *S. boulardii* may display increased adhesiveness then *S. cerevisiae*. In the adhesion essays, the results supported the notion that *S. boulardii* has a stronger capacity to adhere to intestinal epithelial cells than *S. cerevisiae*, a phenotype that may underlie its increased probiotic capacity.

Summarizing, clues for *S. boulardii* superior probiotic properties were found. This strain has a better adaptation to human temperature and perceives less heat and oxidative stress in an ILM medium. *S. boulardii* also showed up-regulation of certain genes associated with probiotic activity when compared with *S. cerevisiae*. This is the case of synthesis of Vas1p 120 kDa protein, polyamine (spermidine) and acetate and the higher expression of certain cell wall and adhesion related genes Cell wall's adhesion proteins up-regulation in *S. boulardii* was confirmed by higher adherence to intestinal epithelium in adhesion assays.

In this thesis, a new functionality was implemented for the ProBioYeastract platform. This new tool finds transcription factors that specifically regulate the inputted genes. This is done by comparison of transcription factor's binding sites (TFBS) within the promoter's sequences and computation of which ones match. Hence, this tool allows a global evaluation of promoter regions in the ProBioYeastract database. As proof-of-principal, this tool was used to analyze differentially expressed genes involved in osmotic and salt stress and ion homeostasis. Yap3p and Gcn4p transcription factors were selected as examples, since they showed potential in affecting gene expression differently in the two strains. Both transcription factors lost certain genes as targets in *S. boulardii*, which might explain its decreased osmotic stress resistance.

It should be noted that, although transcript levels are increased, this does not mean that the transcript products are active. In other words, transcript levels are increased as preparation for potentially needed activity, making the yeast ready to quickly modulate the activity of synthesized proteins at the post-translational level. A differential proteomic expression analysis and a post-

translational modification analysis comparison between *S. boulardii* and *S. cerevisiae* grown in ILM medium and comparison with the results obtained for this thesis' transcriptional analysis would be very interesting.

Analysis of protein Vas1p, spermidine and acetate concentration in the growth medium would also be very interesting in order to corroborate the transcriptional results that appear to show better probiotic properties for *S. boulardii.* In case of high quantities of acetate concentration of the medium, further tests of antimicrobial potential would be highly appealing.

It would also be interesting to assess if the different adhesion capacity of the two strains relies on the increased expression of *HKR1* and *YPS1* genes, observed in planktonic cultivation in intestinal tract like medium, or eventually of other adhesion related genes, whose expression can vary in biofilm formation assays or in the presence of intestinal epithelial cells.

Since the ProBioYeastract new functionality would still take about one hour and fifteen minutes to run using the whole set of differently expressed genes from the transcriptional analysis, a way to speed up the use of this function is still necessary. For that, the matches for each gene could be calculated and stored individually, in order to be easily accessible in the database. It would also be highly interesting to analyze Yap3p and Gcn4p transcription factor activity upon the expression of genes predicted to be differentially regulated in *S. boulardii* versus *S. cerevisiae*, under hyperosmotic and/or salt stress, and compare the results with the obtained with this bioinformatic tool. On the other hand, it would also be interesting to evaluate if an increase in the expression of lost target gene in *S. boulardii*, would also increase its tolerance to osmotic stress and promote an even better probiotic performance of this species.

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