Predicting the mechanisms of probiotic activity in *Saccharomyces boulardii*: a contribution to the development of the ProBioYeastract database

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Biological Engineering

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"Science is the most reliable guide in life."

M. Kemal Atatürk

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ABSTRACT

Saccharomyces boulardii is a well-known probiotic mostly used in pharmaceutical and food industries. Its known functions are mostly related to the prevention and treatment of gastrointestinal diseases. However, the molecular basis of this activity, especially when compared to non-probiotic *S. cerevisiae* strains, remains to be fully established.

This study aimed to evaluate if the registered differences between probiotic and non-probiotic *S. cerevisiae* strains relies on differences at the level of gene transcription regulation. As a result of the *in silico* cross-strain promoter analysis, comparing *S. boulardii* Biocodex and Unique28 strains with *S. cerevisiae* S288C strain, the expression of 26 probiotic-related genes was predicted to be controlled by different transcription factors in probiotic vs non-probiotic strains. Additionally, the pipeline designed for this analysis was used as the basis for a new query in the ProBioYeastract database, whose bioinformatics tools are in construction.

Six selected genes were chosen for differential gene expression analysis, by RT-PCR, in cells grown in YPD medium or YPD with sodium cholate. Among the evaluated genes, *EFG1* and *IMA1* were found to be up-regulated in *S. boulardii* Biocodex, when compared to *S. cerevisiae* BY4741, leading us to propose that their overexpression in *S. boulardii* strains may underly their probiotic activity. Given the importance of *EFG1* in biofilm formation, the ability of *S. boulardii* Biocodex, when compared to *S. cerevisiae* BY4741, to aggregate, adhere to human epithelial cells and form biofilms was evaluated and shown to be higher in all cases.

Altogether, these results suggest that the probiotic activity of *S. boulardii*, when compared to *S. cerevisiae*, is, at least, partially due to its higher ability to form biofilm, and adhere to epithelial surfaces, that may in part rely in the up-regulation of the *EFG1* gene.

Keywords: ProBioYeastract, S. *boulardii*, *S. cerevisiae*, probiotic, biofilm formation, adhesion, aggregation, gene expression, *FLO5*, *EFG1*, *TGL4*, *YDC1*, *SPE2*, *IMA1*.

RESUMO

Saccharomyces boulardii é um probiótico bem conhecido, usado principalmente nas indústrias farmacêutica e alimentar. As suas funções conhecidas estão maioritariamente relacionadas com a prevenção e tratamento de doenças gastrointestinais. Contudo, a base molecular subjacente a esta actividade permanece por esclarecer, especialmente quando comparada com estirpes não-probióticas de *S. cerevisiae*.

Este estudo tem como objectivo avaliar se as diferenças registadas entre estirpes probióticas e não-probióticas de *S. cerevisiae* se baseiam em variações ao nível da regulação da transcrição. Como resultado da análise comparativa *in silico* de promotores de genes homólogos de *S. boulardii* Biocodex, *S. boulardii* Unique28 e *S. cerevisiae* S288C, foi possível prever que a expressão de 26 genes, com uma relação prevista com a actividade probiótica, é controlada por factores de transcrição diferentes, em estirpes probióticas em comparação com não-probióticas. Addicionalmente, a sequência de passos definida para esta análise in silico foi usada como base para o desenvolvimento de uma nova ferramenta na base de dados ProBioYeastract, cujas ferramentas bioinformáticas estão ainda em construção.

Foram seleccionados seis genes para análise de expressão diferencial, por RT-PCR, em células cultivadas em meios YPD e YPD+colato de sódio. Dentre os genes analisados, verificou-se que o *EFG1* e o *IMA1* são sobre-expressos em *S. boulardii* Biocodex, em comparação com *S. cerevisiae* BY4741. Esta observação permite propôr que a sua sobre-expressão em estirpes de *S. boulardii* pode estar subjacente à sua actividade probiótica. Dada a importância do gene *EFG1* na formação de biofilme, foi confirmada a maior capacidade de agregação, adesão a epitélio humano e formação de biofilme de *S. boulardii* Biocodex, em comparação com *S. cerevisiae* BY4741.

Em conjunto, os resultados obtidos sugerem que a actividade probiótica de *S. boulardii*, em comparação com *S. cerevisiae*, se deve, pelo menos em parte, à sua maior capacidade de formação de biofilme e de adesão a células epiteliais, que poderá estar relacionada com a sobre-expressão do gene *EFG1*.

Palavras-chave: ProBioYeastract, S. *boulardii*, *S. cerevisiae,* probióticos, formação de biofilme, adesão, agregação, expressão génica, *FLO5*, *EFG1*, *TGL4*, *YDC1*, *SPE2*, *IMA1*.

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ABBREVIATIONS

WHO: World Health Organization

FDA: American Food and Drug Administration

AAD: Antibiotic-Associated Diarrhea

CDI: *Clostridium difficile* infection

TD: Traveler's diarrhea

LPS: Lipopolysaccharide

IBS: Irritable bowel syndrome

CD: Celiac disease

ST: *Salmonella typhimurium*

MAPK: Mitogen-activated protein kinase

YPD: Yeast-extract-peptone-dextrose

SDB: Sabouraud's dextrose broth

RPMI: Roswell Park Memorial Institute

CFU : Colony forming units

cDNA :complementary DNA

RT-qPCR : quantitative real time polymerase chain reaction

DEPC : diethylpyrocarbonate

EDTA : Ethylenediamine tetraacetic acid

Sb: Saccharomyces boulardii

Sc: Saccharomyces cerevisiae S288C

YEASTRACT: Yeast Search for Transcriptional Regulators And Consensus Tracking

SI: Sucrose-Isomaltase

MGA: Maltase-glucoamylase

ANP: Aminopeptidase N

LPH*:* Lactase-phlorizin hydrolase

IAP: Intestinal Alkaline Phosphatase

TFBS: Transcription Factor Binding Sites

1. INTRODUCTION

1.1. Definition of probiotic

The word Probiotic comes from a Latin/Greek root and means literally "for life". In 1857, Pasteur discovered lactic acid bacteria for the first time. However, it was Élie Metchnikoff that became known as the father of probiotics since he asserted that lactic acid bacteria induced lower pH in the colon due to the breaking down of lactose, thus inhibiting the growth of proteolytic bacteria (Ozen and Dinleyici, 2015). The word Probiotic was coined by Fuller and defined as a "live non-pathogenic microbial feed or food supplement which beneficially affects the host by improving its intestinal microbial balance" (Czerucka and Rampal, 2002).

Nowadays, according to the World Health Organization (WHO), Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit to the host." (Hunt *et al.,* 2017).

Based on this premise, there has been an increasing trend of using probiotic organisms worldwide to contribute to human health, particularly as co-adjuvants in the treatment of human diseases (Fig 1). Many of these probiotic organisms contribute by either substituting or aiding the re-establishment of the natural gastrointestinal flora, or microbiota (Table 1).

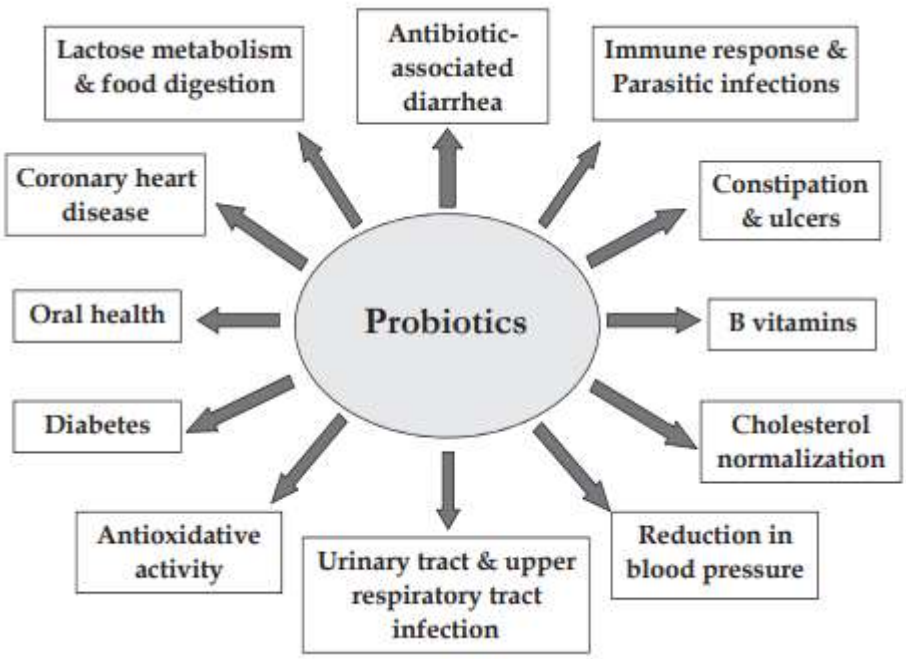


Figure 1. Major benefits of probiotics for human health (Nagpal *et al.*, 2012)

Table 1. Mostly used probiotic microorganisms in the pharmaceutical and food industry (Holzapfel *et al.*, 2001)

Lactobacilli sp	***Bifidobacterium sp***	***Bacilli sp***	***Saccharomyces sp***
Lactobacillus acidophilus	*Bifidobacterium bifidum*	*Bacillus coagulans*	*Saccharomyces boulardii*
Lactobacillus casei	*Bifidobacterium longum*	*Bacillus subtilus*	
Lactobacillus bulgaricus	*Bifidobacterium infantis*	*Bacillus clausii*	
Lactobacillus rhamnosus	*Bifidobacterium animalis*		

Some of the conditions that need to be met in order for a specific microbial strain to be called Probiotic are the following (Vandenplas, Huys and Daube, 2015) ;

**In vitro* studies: to show potential probiotic activity

*Assessment of safety: to indicate that the strain carries no human or environmental toxicity

**In vivo* studies: to indicate probiotic activity, that is a positive health impact in the target host

*Good probiotic properties (Daliri and Lee, 2015), which may include:

- Resistance to pancreatic enzymes, low pH and bile which provides survival during passage through the intestinal tract, an important property for oral administration
- Adhesion to the intestinal mucosa; pathogen exclusion, preventing its adhesion and colonisation; enhancing damaged mucosa recovery; prolonged transient colonization (Kechagia *et al.*, 2013)
- Having human origin which means being a natural human commensal
- Proven, through clinical evidence, to induce positive health effects
- Having good technological features for industrial manufacturing, which include strain stability; oxygen tolerance, and short generation time (Fietto *et al.*, 2004)
- Production of antimicrobial compounds, active against pathogens, such as organic acids, hydrogen peroxide and bacteriocins (Gut *et al.*, 2018)

Probiotics should be clinically validated and documented health effects of minimum effective dosage in products should be available. They should also be classified as Generally Recognised

As Safe 'GRAS', an American Food and Drug Administration (FDA) designation for chemicals or food additives considered safe by experts. This will imply a previous 'history of safe use' and safety in food. Moreover, they should be non-invasive, non-carcinogenic and non-pathogenic to human (Gut *et al.*, 2018; Kechagia *et al.*, 2013).

1.2. *Saccharomyces boulardii* as a probiotic

Saccharomyces boulardii also called *Saccharomyces cerevisiae* var. *boulardii*, was isolated by the French scientist Henri Boulard in 1920 from the skin of lychee and mangosteen in Indochina, during a cholera outbreak (Edwards-Ingram *et al.*, 2007; Batista *et al.,* 2014).

Saccharomyces boulardii is a well-studied probiotic yeast known as a therapeutic agent for the prevention of recurrence of several gastrointestinal diseases, which are mainly grouped into acute and chronic. Acute diseases include Antibiotic-associated diarrhea (AAD), *Clostridium difficile* infection (CDI), and Acute diarrhea, including that caused by Rotavirus infection in children, Persistent diarrhea, Enteral nutrition-related diarrhea, Traveler's diarrhea (TD), and *Helicobacter pylori* infection. On the other hand, chronic diseases include Crohn's disease, Ulcerative colitis and Irritable bowel syndrome (IBS) (Kelesidis and Pothoulakis, 2012).

Compared to bacterial probiotics, *S. boulardii* is naturally resistant against all kinds of antibiotics, given its eukaryotic nature (Czerucka, Piche and Rampal, 2007; Graff *et al.*, 2008; Kelesidis and Pothoulakis, 2012).

A number of studies, conducted *in vitro, in vivo*, or as clinical or meta-analysis, have shown that *S. boulardii* is a probiotic, having a positive impact in the treatment and prevention of several diseases of the gastrointestinal tract as summarized in Table 2.

Table 2. Summary of the major findings on the probiotic efficacy of *S. boulardii*, according to *in vitro, in vivo*, clinical and meta-analysis studies for children and adult patients. EHEC- Enterohemorrhagic *Escherichia coli*, IL-8 Interleukin 8, IL-6 Interleukin 6, IL-1β Interleukin 1 β, TNF-α Tumor Necrosis Factor-alpha, IFN-γ Interferon Gamma, CDI- *Clostridium difficile* infection, AAD Antibiotic-Associated Diarrhea, TD Traveler's diarrhea PC-Placebo controlled, PG- Parallel-group, R- randomized, Ab-antibiotic, NA-No Available.

	Disease	Type of Study	Number of Target group	Dose& Duration	Major findings	References
In vitro	Diarrhea caused by Enterohemorrhagic Escherichia coli (EHEC) infection	Study in culture media	x	x	The protective effect on EHEC infection reduced expression of pro-inflammatory cytokines (IL-8, IL-6, IL-1β, TNF-α, and IFN-γ)	(Dahan *et al.*, 2003)
	Diarrhea caused by EHEC infection	Study in culture media	x	x	*S. boulardii* reduced in TNF-α and related apoptosis in EHEC infected T84 intestinal epithelial cells	(Dalmasso *et al.*, 2006)
	Diarrhea	Study in culture media	x	x	Protective effects against diarrheal pathogens by reducing the pro-inflammatory response	(Fidan *et al.*, 2009)
In vivo	CDI	An animal study (in rat and rabbit ileal loop)	x	NA	Removed toxin receptors with protease activity, decreased brush border glycoproteins	(Pothoulakis *et al.*,1993)

	CDI	An animal study (in rat)	x	x	Reduced the *C. difficile* colitis, enhanced the intestinal mucosal immune response	(Castagliuolo *et al.,* 1998)
Clinical studies	AAD	DP, PC, PG	193 Adult patient	1g/d receiving duration of Ab+2 wk	AAD rate is decreased	(McFarland *et al.,* 1994)
	AAD	MC,P	367 Adult Patient	500 mg twice daily	AAD rate is decreased	(Duman *et al.*, 2005)
	H. pylori Infection	P, R, PC	124 Adult patient	14 d TT+Ab for 10 d+1 g/d for 28 d	A significant decrease in recurrences	(Cindoruk *et al.*, 2007)
	CDD	P, R, PC	82 Adult patient	1g /d Ab(for 10 d)+P for 28 d)	Decreased recurrence, no adverse effect observed	(Surawicz *et al.*, 2000)
	ADD	R, DP, PC	269 children 6 mo to 14 yr	250 mg	Decreased risk of antibiotic-associated diarrhea caused by C. *difficile* no adverse effect observed	(Kotowska, Albrecht and Szajewsk, 2005)
	AAD	PC	653 children 1-15 year	NA	Reduced rate of diarrhea	(Erdeve, Tiras and Dallar, 2004)
	Rota-virus infection	DP, PC	200 children 3 mo-7 yr	250 mg/d 7 day	Decreased duration of diarrhea and hospitalization	(Kurugöl and Koturoğlu, 2005)
Meta-analysis	Traveler's Diarrhea (TD)	x	5029 study patients	250-1000 mg/day, During of trip, 3wk	Effective in the prevention of TD	(McFarland, 2007)

AAD	x	5029 study patients	500-1000 mg/d During Ab with additional, 3 days to 2 weak after	Effective for the prevention of AAD with a daily dose > 10^9 cells	(McFarland, 2010)
AAD	x	4780 participants	x	Effective in reducing the risk of antibiotic-associated diarrhea in children and adults.	(Szajewska and Kołodziej, 2015)

Recent evidence have shown that probiotics communicate with the host by modulating key signaling pathways, for example, NFκB and Mitogen-activated Kinases (MAPK) pathways, but the molecular mechanisms by which expression of proteins produced by probiotics could participate in Gastro-Intestinal Tract (GIT) homeostasis are still unclear. Additionally, probiotics can change the physiology of the microbiota, but the underlying mechanisms are also unclear at the molecular level. Understanding these mechanisms of action will help to develop better prophylaxis and therapeutic strategies. The following section underlines current knowledge on the mechanisms of *S. boulardii* against human diseases, while suggesting the genes that may underlie its activity, as summarized in Table 3.

1.3. Clinical efficacy of *S. boulardii* as a probiotic:

1.3.1. Against Acute Diarrhea

Diarrhea is a widespread health problem all over the world. It generally is diagnosed when observing mushy or watery stool, per-day stool weight of >200 g, or stool frequency of more than three per day (Högenauer *et al.*, 1998). There are a lot of *in vitro, in vivo*, clinical studies indicating the efficacy of *S. boulardii* probiotic to reduce acute diarrhea as indicated in Table 2.

1.3.1.1. Against Antibiotic-Associated Diarrhea (AAD)

Antibiotic-associated diarrhea is defined as "otherwise unexplained diarrhea that occurs in association with the administration of antibiotics"(Varankovich *et al.,* 2015). AAD leads to osmotic diarrhea, caused by suppression of anaerobic bacteria, a decrease in carbohydrate metabolism, disruption of protective effects of commensal bacteria and alleviation of colonic mucosal resistance to pathogenic bacteria, finally resulting in dysbiosis (altered microbiota). *S. boulardii* is a well-known kind of probiotic yeast that can mostly relieve antibiotic-associated diarrhea (Surawicz *et al.*, 1989; Kotowska, Albrecht and Szajewsk, 2005; Szajewska and Kołodziej, 2015).

The causes of AAD can be rotavirus infection in children (Kurugöl and Koturoğlu, 2005), *C. difficile, Candida spp* and *Salmonella spp* infection. Associated to AAD, disturbance caused by allergic and toxic effects of antibiotics on intestinal mucosa has also been registered (Högenauer *et al.*, 1998).

1.3.1.2. Against Rotavirus Infection (in children)

Acute Rotavirus infection is AAD that targets mostly children. Kurugol et al. (2015) suggested in a double-blind placebo-controlled study that *S. boulardii* has a significant effect on the duration of acute diarrhea, and hospital stay in children (Kurugöl and Koturoğlu, 2005).

1.3.1.3. Against *Clostridium difficile* Infection (CDI)

Clostridium difficile is a known spore-forming, anaerobe, and gram-positive bacterium. (Khan and Elzouki, 2014; Seekatz and Young, 2014). Its spores are very resistant to severe environmental conditions. This type of infection typically causes antibiotic-associated diarrhea and pseudomembranous colitis (Peniche, Savidge and Dann, 2013). The main risk factors of CDI include intensive usage of antibiotics, old age, multiple co-morbid conditions, long stays in hospitals, etc. (McFarland, 2006; Predrag, 2016).

Clostridium difficile produces 2 main toxins, Toxins A (enterotoxin) and B (cytotoxin) (Pothoulakis, 2009). These toxins are responsible for *C. difficile* pathogenesis that leads to increasing Regulatory T cells (Tregs). Toxin release leads to the production of secretory IgA (slgA), inflammatory cytokines and neutrophils in the gut to maintain homeostasis as summarized in Fig 2.

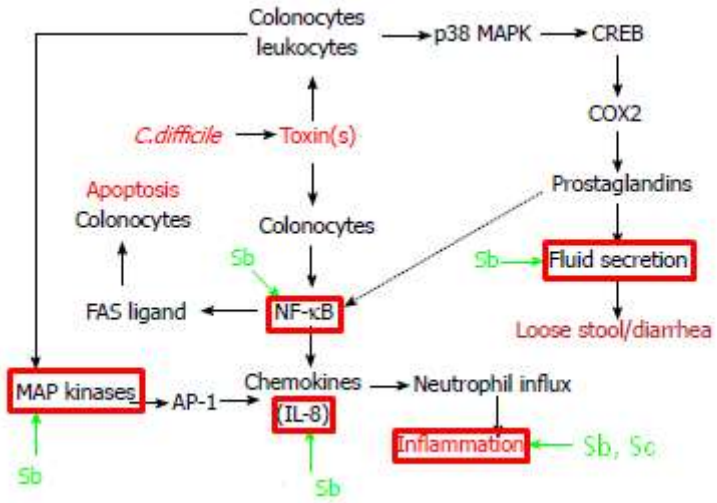


Figure 2. The mechanism of Action of *S. boulardii* against the consequences of *C. difficile* infection (Adapted from Fitzpatrick 2013)

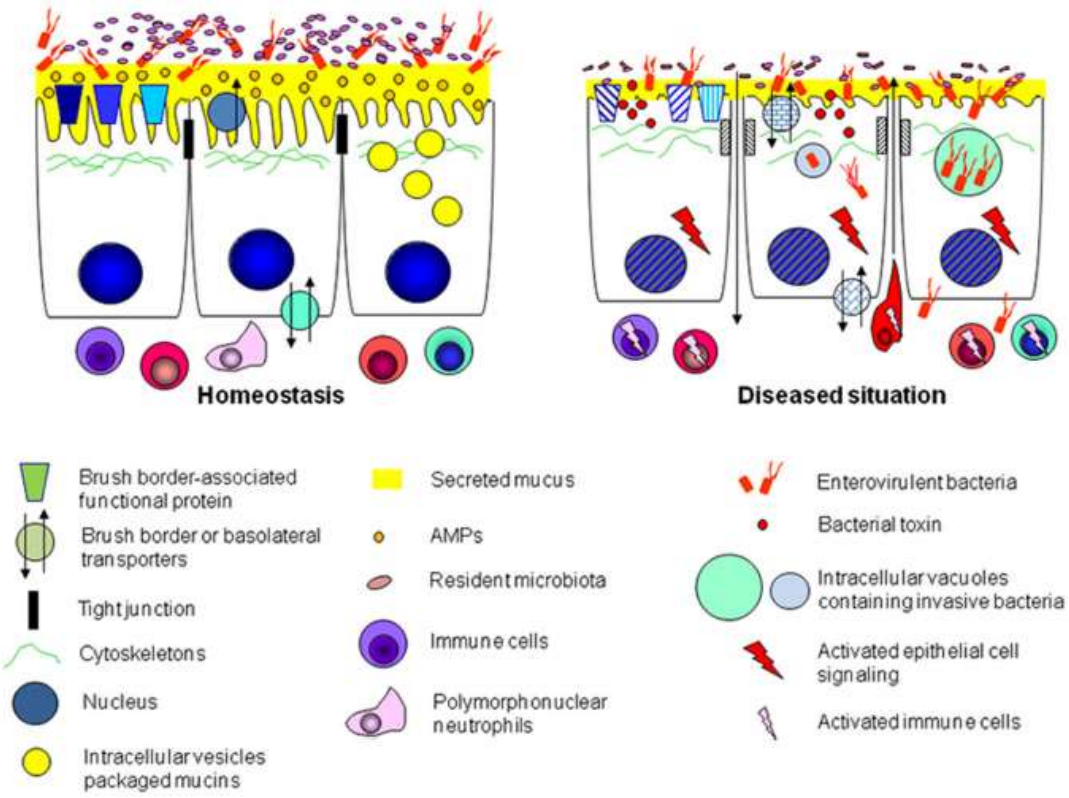


Figure 3. Representation of the healthy balance between intestinal epithelial cells and the microbiota (homeostasis) and disease consequences on this balance (dysbiosis) (Moal and Servin, 2013)

Saccharomyces boulardii (*Sb*) oral administration in gnotobiotic mice, was observed to significantly decrease the mortality caused by *C. difficile* infection (dysbiosis) as shown in Figure 3. Indeed, a single dose of *Sb* preserved 16% of mice, whereas 56% were protected when *Sb* was administrated continuously in the drinking water. Although direct inhibition of *C. difficile* numbers was not detected, reduced toxin production was indicated in the study (Chen, Dong and Sun, 2013).

One clinical study among 168 patients demonstrated that decreasing recurrence of CDI was favored in patients treated with high-dose vancomycin (2 g/day) supplemented with *S. boulardii* when compared to the use of the same antibiotic dosage plus placebo (Surawicz *et al.*, 2000).

Chen et al showed that *S. boulardii* reduced the activity of *C. difficile* toxin A-associated enteritis by blocking the activation of Erk1/2 MAP kinases. ERK and p38 MAP kinases are induced by *C. difficile* toxin A. They are needed for IL-8 gene expression cell necrosis. Moreover, they demonstrated that *Sb* defends against intestinal inflammation and modulates host inflammatory signaling pathways to exert its beneficial effects (Chen *et al.*, 2006).

1.3.1.4. Against Diarrhea caused by *Helicobacter pylori*

Helicobacter pylori is a spiral, microaerophilic, gram-negative bacterium with flagella (Kamboj, Cotter, and Oxentenko 2017), and the causative agent of gastric and duodenal ulcers, being a risk factor for gastric malignancies. *Helicobacter pylori* use urease to gain access to epithelial cells by increasing the pH which in turn lowers mucus viscosity allowing the organism to propel itself through the mucus layer that coats the stomach wall.

Homan and Orel (2015) reported that *S. boulardii* possess neuraminidase activity. This activity by *S. boulardii* removed from the surface α (2-3)- linked sialic acid, the ligand for the sialic acid-binding *H. pylori* adhesion (Homan and Orel, 2015).

1.3.2. Against Irritable Bowel Syndrome (IBS)

Irritable bowel syndrome (IBS) is a chronic gastrointestinal problem, associated with chronic abdominal pain and discomfort. Other symptoms of IBS are abdominal distension, bloating and flatulence, straining, and urgency (Distrutti *et al.*, 2016; Spiller *et al.,* 2007). There is not enough data in clinical studies to confirm the efficacy of *S. boulardii* against this syndrome, but *in vivo* and *in vitro* studies suggest as much (Sivananthan and Petersen, 2018). In a randomized clinical trial study for irritable bowel syndrome, the group treated with *S. boulardii* exhibited higher levels of pro-inflammatory cytokines interleukin-8 (IL-8), tumor necrosis factor-α, anti-inflammatory cytokines IL-10 and IL-10/IL-12 ratio, and significantly lower levels of human blood and tissue cells (Distrutti *et al.*, 2016). *S. boulardii* regenerates lymphocytes such as B lymphocytes, NK cells, and T cells in a model of chronic IBD. This effect is illustrated in Fig 4.

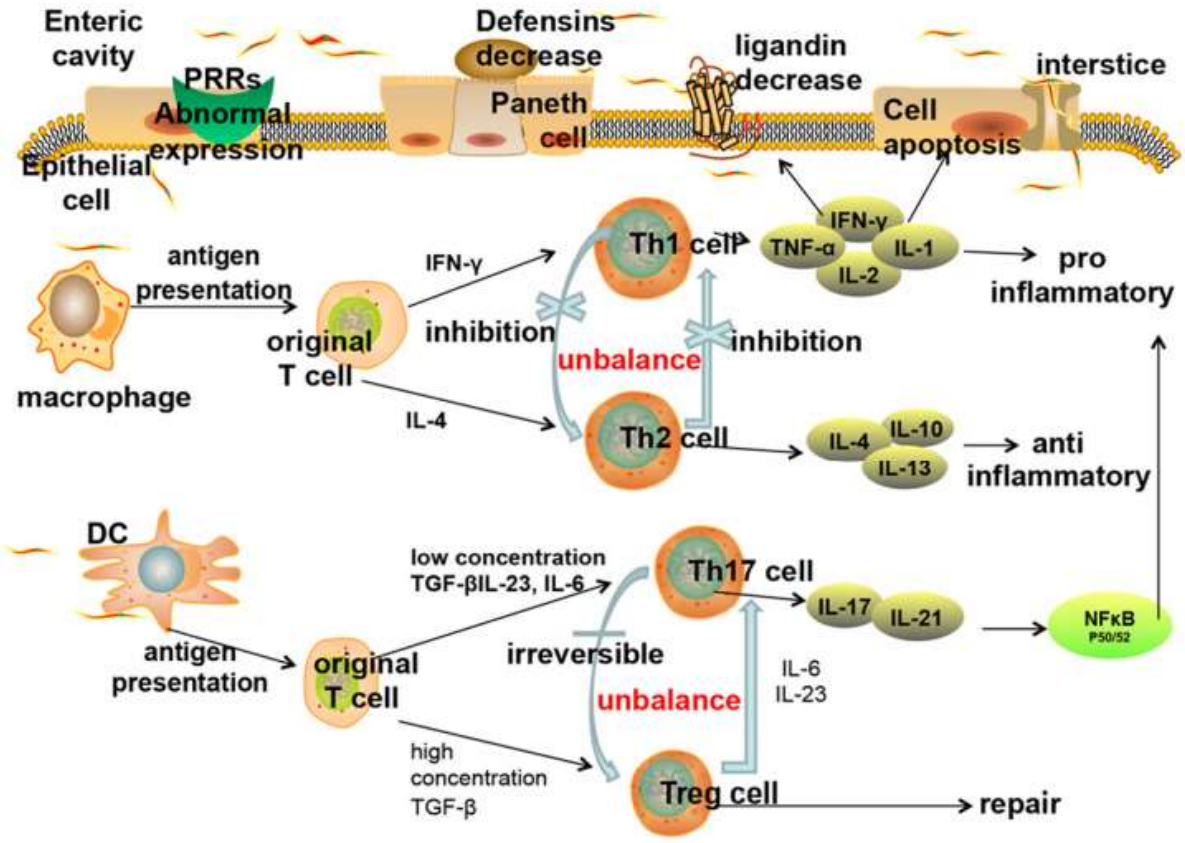


Figure 4. The mode of action of probiotic for Inflammatory bowel disease (Huang and Chen, 2016).

Moreover, production of high levels of NO leads to inflammatory effects in IBD because NO is released through the conversion pathway of L-arginine to NO and L-citrulline. Three isoforms of the nitric oxide synthase (NOS) catalyze these reactions. These are Neuronal NOS, endothelial NOS and inducible NOS (iNOS) (Zanello *et al.*, 2009). One *in vivo* study shown that *S. boulardii* inhibits iNOS activity in the rat castor oil-induced diarrhea model (Girard, Pansart and Gillardin, 2005).

1.3.3. Against Metabolic Diseases: lactose and gluten intolerance and obesity

Recent studies have shown that probiotics play an important role in the regulation of energy homeostasis, metabolic inflammation, lipid metabolism, and glucose metabolism as shown in Figure 5 (as reviewed in Moré and Vandenplas 2018). *S. boulardii* probiotic activity against metabolic diseases, such as lactose and gluten intolerance, is thought to be due to the supply of digestive enzymes or induction of their expression by epithelial cells as followed:

Sucrose-Isomaltase (SI) - SI displays α-glucosidase activity, hydrolyzing oligomers with (1→6)-α-d- glucosidic linkages including sucrose. (Bernasconi, Craynest and Maldague, 1986) A recent study reported that *S. boulardii* leads to the upregulation of sucrase-isomaltase expression in intestinal cells. *S. boulardii* also produces SIs, encoded by the *IMP1*, *IMP2*, and *IMP5* genes, influencing palatinose metabolism. Amongst these iso-maltases, Imp1 and Imp2 possess high affinity to palatinose. Interestingly, unlike *S. cerevisiae*, *S. boulardii* has no *IMP3* and *IMP4* genes, and in some strains not even *IMP2* (Khatri *et al.*, 2013, 2017).

Maltase-glucoamylase (MGA) – the expression of α-glucosidase, containing 2 domains with differing substrate specificity on maltose/starch and glucose oligomers with α(1→4) bonds, is up-regulated upon exposure to *S. boulardii* (Bernasconi, Craynest, and Maldague 2002; Zaouche *et al.,* 2000).

Aminopeptidase N (ANP) - known as alanyl aminopeptidase, or neutral brush border aminopeptidase, these enzymes digest peptides generated from hydrolysis of proteins by gastric and pancreatic proteases. The expression of these host proteins is stimulated upon exposure to *S. boulardii* (Khatri *et al.,* 2017; Zaouche *et al.,* 2000; Moré and Vandenplas 2018).

Lactase-phlorizin hydrolase (LPH) - known as a digestive enzyme, LPH has two domains, one splitting, among others, lactose, cellobiose o-nitrophenyl- β-glucopyranoside, and o-nitrophenyl- β-galactopyranoside, and the other splitting, among others, phlorizin, β-glycopyranosylceramides, and m-nitrophenyl-β-glucopyranoside. The expression of these host proteins is stimulated upon exposure to *S. boulardii* (Bernasconi, Craynest and Maldague, 1986).

Intestinal Alkaline Phosphatase (IAP) – IAP is a protein expressed by the intestinal epithelium to protect gut homeostasis. IAP dephosphorylates lipopolysaccharides derived from the cell wall of gram-negative bacteria, preventing transmigration of bacteria across the epithelium; it also dephosphorylates other potentially pro-inflammatory ligands. In addition, its functions are detoxification of bacterial endotoxins, dephosphorylation of Tri- and Di-Phosphorylated nucleotides, regulation of the intestinal microbiome and lipid absorption. When IAP expression is decreased, it leads to increased intestinal inflammation, dysbiosis, bacterial translocation and subsequently systemic inflammation (Moré and Vandenplas 2018).

IAP plays an important role in the intestine encompasses both protection from systemic infections and chronic inflammatory diseases (Estaki, DeCoffe and Gibson, 2014).

Interestingly, *S. boulardii* stimulates intestinal alkaline phosphatase (IAP) expression, offering treatment for gluten intolerance, and obesity (Moré and Vandenplas, 2018).

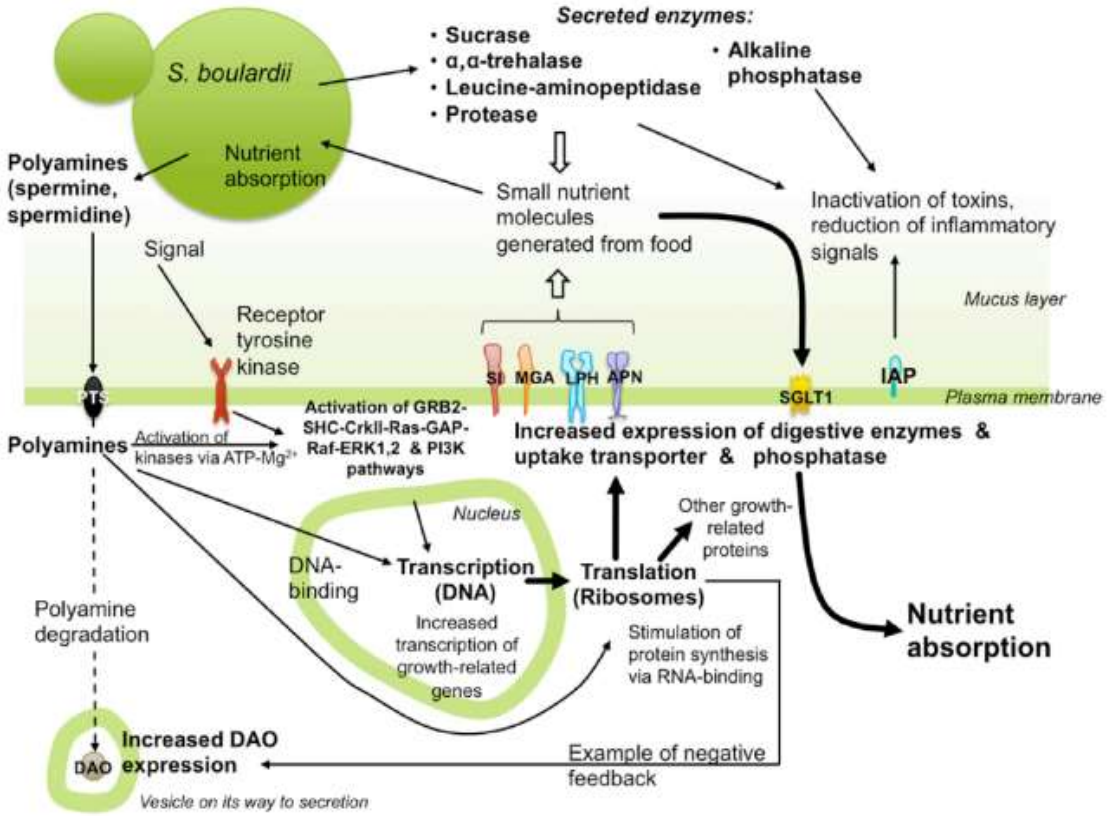


Figure 5.Summary of digestive enzymes stimulated or produced by *S. boulardii* (Moré and Vandenplas, 2018)

1.3.3.1. Against Gluten Intolerance or Celiac Disease

Celiac disease (CD) is a malabsorptive enteropathy, triggered by an inappropriate T cell-mediated immune response to dietary gluten proteins. After ingestion of glutens, transforming into gliadin peptides reach the subepithelial region of the intestinal mucosa. Glutenins are also involved in T cell response and trigger an inappropriate T cell-mediated immune response, which might result in intestinal mucosal inflammation and extraintestinal manifestations. In addition, Gliadins and glutenins includes a high content of proline (15%), hydrophobic amino acids (19%), and glutamine (35%), so they are named prolamins. Because of this glutamine- and proline-rich structure, gluten proteins are resistant to complete digestion by pancreatic and brush border proteases (Caputo *et al.*, 2010). As a result of this, proline mechanism play a key role in balancing microbiota.

There are two important pathways for celiac disease: one is the direct impact on the epithelium that includes the innate immune response, the other involves the adaptive immune response involving $CD4^+$ T cells in the lamina propria that recognize processed gluten epitopes. High levels of pro-inflammatory cytokines are produced by activated gliadin-specific $CD4^+$ T cells. Therefore, stimulating a Th1 response results in mucosal remodeling and villous atrophy. When IgA-deficiency is determined in patients, they are generally asymptomatic, this situation leading to the development of gastrointestinal disorders such as celiac disease (CD) and allergies (Mantis *et al.,* 2011) as shown in Fig 6.

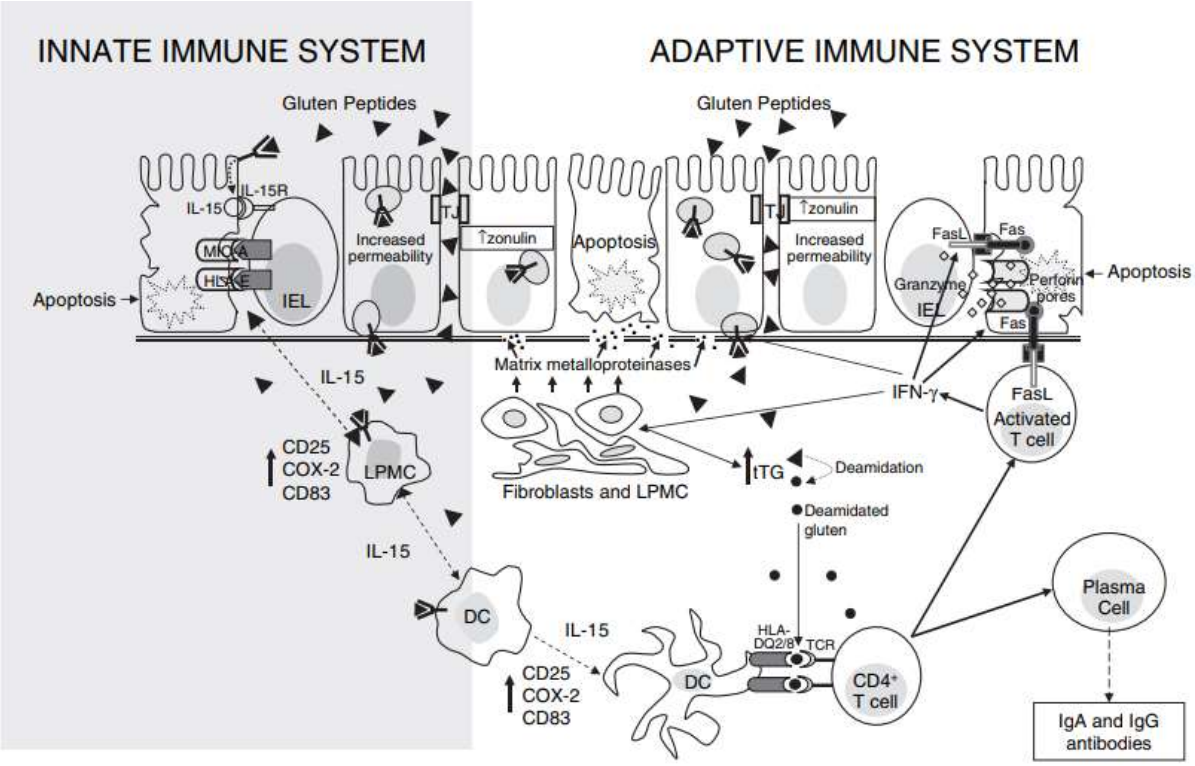


Figure 6. Mechanism of celiac disease (Ciccocioppo *et al.*, 2005)

Cristofori et al. (2018) reported that *Saccharomyces boulardii* KK1 strain might be able to hydrolyze the gliadin toxic peptides, and its consumption was followed by improved enteropathy and a decrease of histological damage and pro-inflammatory cytokine production, in a model of gluten sensitivity in BALB/c mice (Cristofori *et al.*, 2018).

1.3.3.2. Against Lactose intolerance or malabsorption

Lactose intolerance or lactose malabsorption is a disorder related to the lack of enzymes required to degrade lactose, including lactases or beta-galactosidases, into glucose and galactose as indicated in Fig 7. Within this context, Oak and Jha (2018) reported that *S.*

boulardii increases the activity of intestinal enzymes, for example, disaccharidases, α-glucosidases, alkaline phosphatases, and aminopeptidases.

Primary lactase deficiency occurs when the body produces considerably less lactase, and it can only break down smaller amounts of lactose. Secondary lactase deficiency occurs when less lactase is produced as an indirect consequence of bowel problems or chronic inflammation, such as those associated with gluten intolerance or Crohn's disease (Oak and Jha, 2018).

Bacterial enzymes degrade undigested lactose in the large bowel, leading to osmotic diarrhea. Other symptoms of lactose deficiency are bloating, feeling full, pain and abdominal discomfort, flatulence, and a condition called irritable bowel syndrome (IBS).

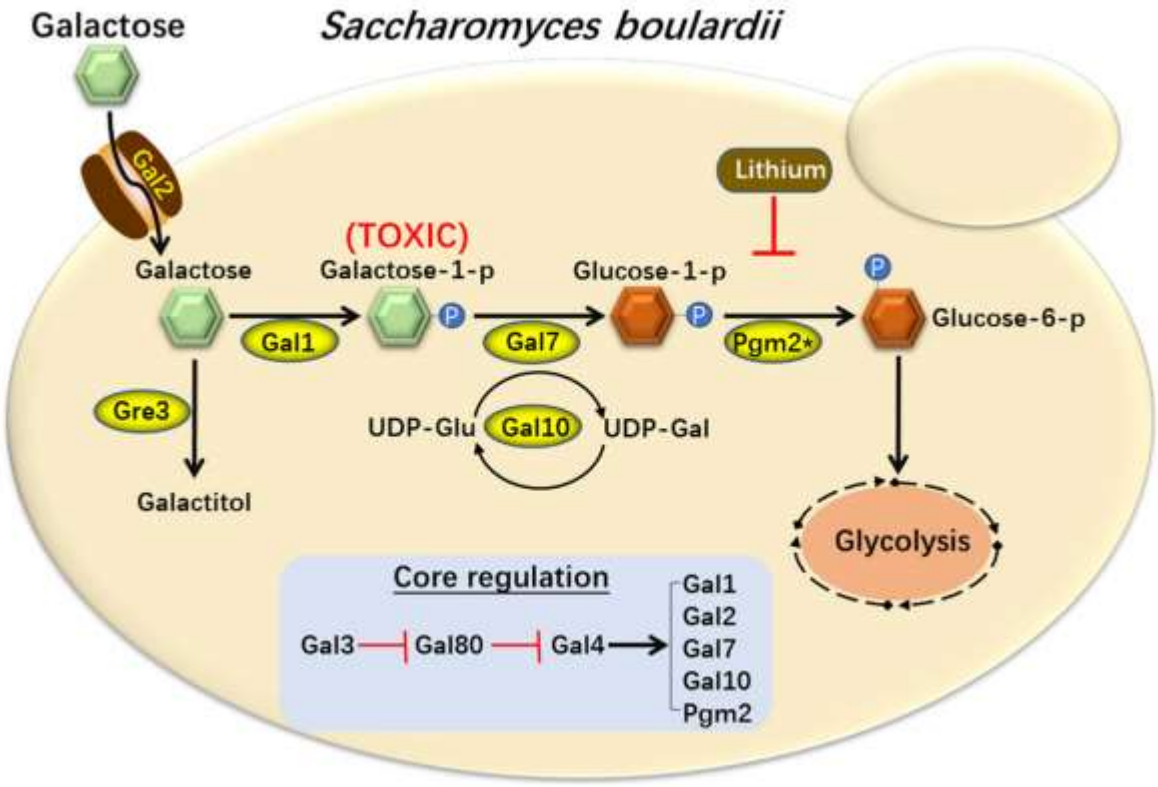


Figure 7. Schematic pathway representation of galactose utilization and its related genes in *S. boulardii* (Liu et al., 2018)

S. boulardii rises the intestinal absorption of D-glucose that might enhance uptake of water and electrolytes during diarrhea (Oak and Jha, 2018).

1.3.3.3. Against Obesity & Type II diabetes

Obesity is defined as abnormal or excessive fat distribution (Kobyliak *et al.*, 2016). In this metabolic disease, lipid metabolism plays an important role. Interestingly, an *in vivo* study

showed that when *S. boulardii* was administered daily by oral gavage in obese and type II diabetic mice during 4 weeks, mice displayed decreased body weight gain and fat mass. It also decreased the hepatic steatosis or fatty liver, which means buildup fat in the liver, and total liver lipids content, systemic inflammation and plasma cytokine concentrations of IL-6, IL-4, IL-1β, and TNF-α. in obese mice (Everard *et al.*, 2014). According to this study, *S. boulardii* might be a favorable co-adjuvant to treat obesity and Type II diabetes.

Fatty acids are building blocks for the synthesis of membrane lipids (phospholipids, sphingolipids) and storage lipids (triacylglycerols, steryl esters). Moreover, phospholipids, sterols, and sphingolipids are essential components of cellular membranes. Intestinal short chain fatty acids (SCFAs) and their link provide healthy microbiota in the human body. Thus, secreting the SCFAs by *S. boulardii*, is believed to be linked to obesity diseases. Fatty acid metabolism-related genes in yeast *Saccharomyces cerevisiae* as indicated in Fig 8.

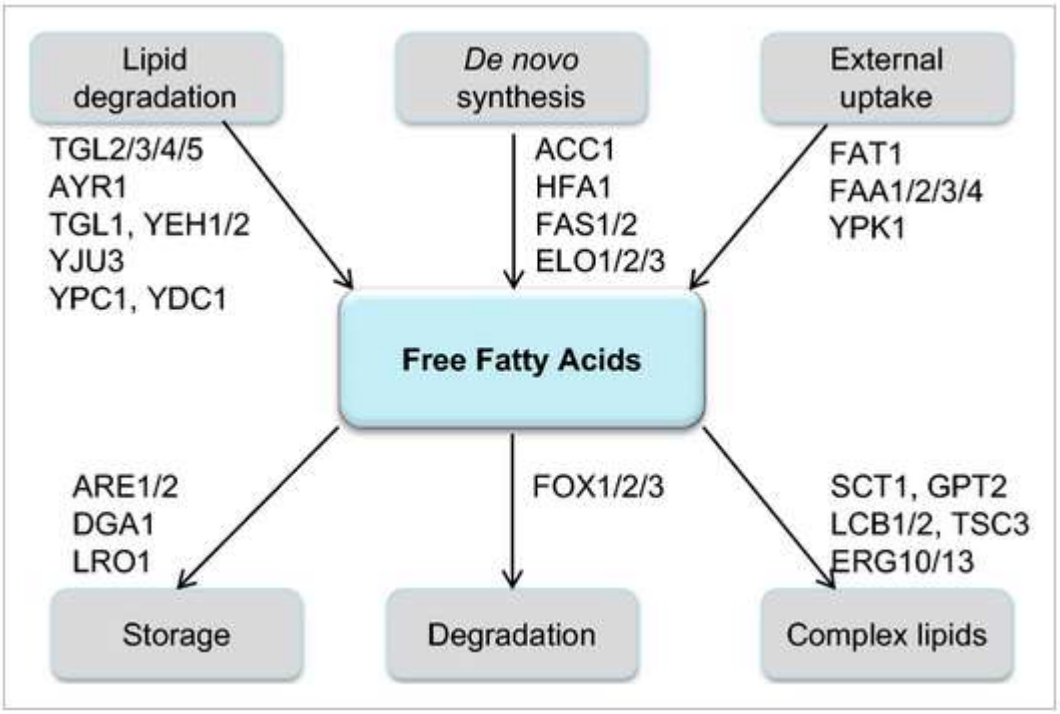


Figure 8.Fatty acid metabolism in *S. cerevisiae* (Klug, 2014)

1.4. Mode of Action for *S. boulardii* as Probiotic

The possible mechanisms of probiotic activity in intestinal inflammatory diseases for therapeutic agents include the following properties (Figure 9);

- Antagonism against enteric pathogens (antimicrobial effect, toxin deactivation, etc)

- Enhancement of the gut mucosal barrier (digestibility, nutritional value, secreting of SCFAs, etc)
- Inhibition or enhancing of local secretion of inflammatory mediators (anti-inflammatory and pro-inflammatory)
- Stabilization of local immunological activity (Immunomodulation effect, etc)
- Quorum sensing (Adhesion, aggregation, biofilm formation, etc)

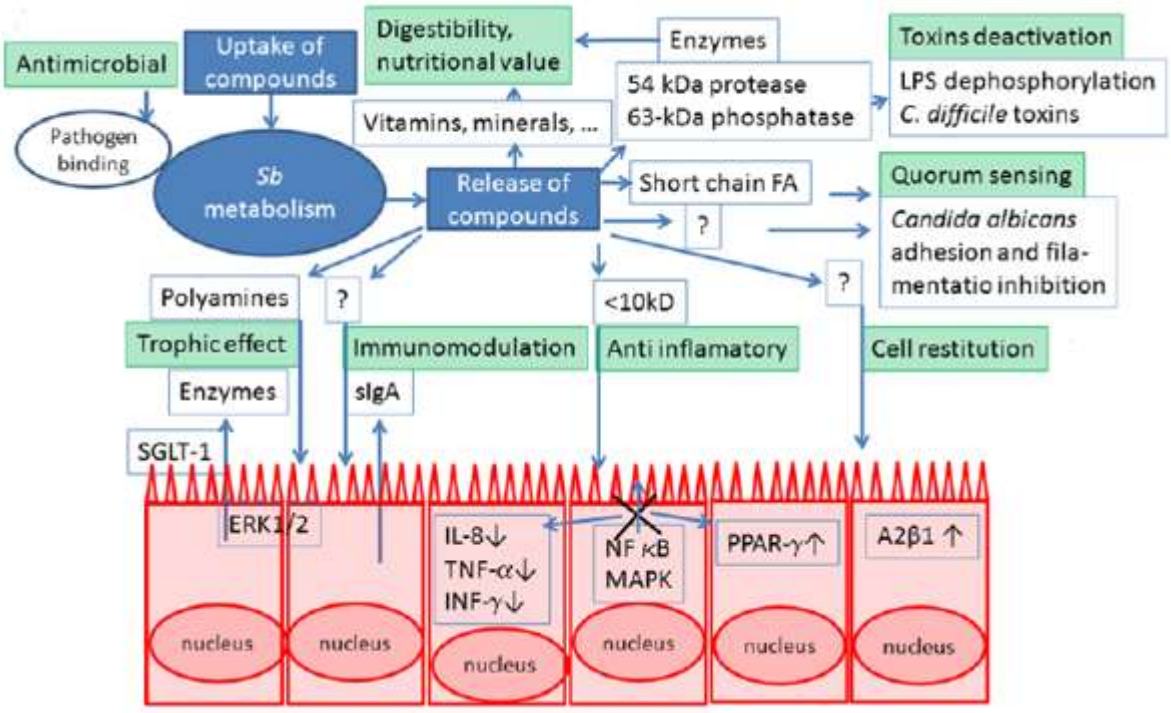


Figure 9. Schematic representation of the possible mode of action for *S. boulardii* (Adapted from Łukaszewicz, 2012)

The mode of action of *S. boulardii* can be mainly categorized as having luminal action, trophic action and mucosal action (McFarland, 2010).

1.4.1. Luminal Action

Intestinal epithelial cells are generally classified as enterocytes, paneth cells and goblet cells as shown in Fig 10. The main functions of the epithelium are to form a selective barrier in the intestine walls and to support nutrient and water transport while protecting from microbial contamination of the interstitial tissues.

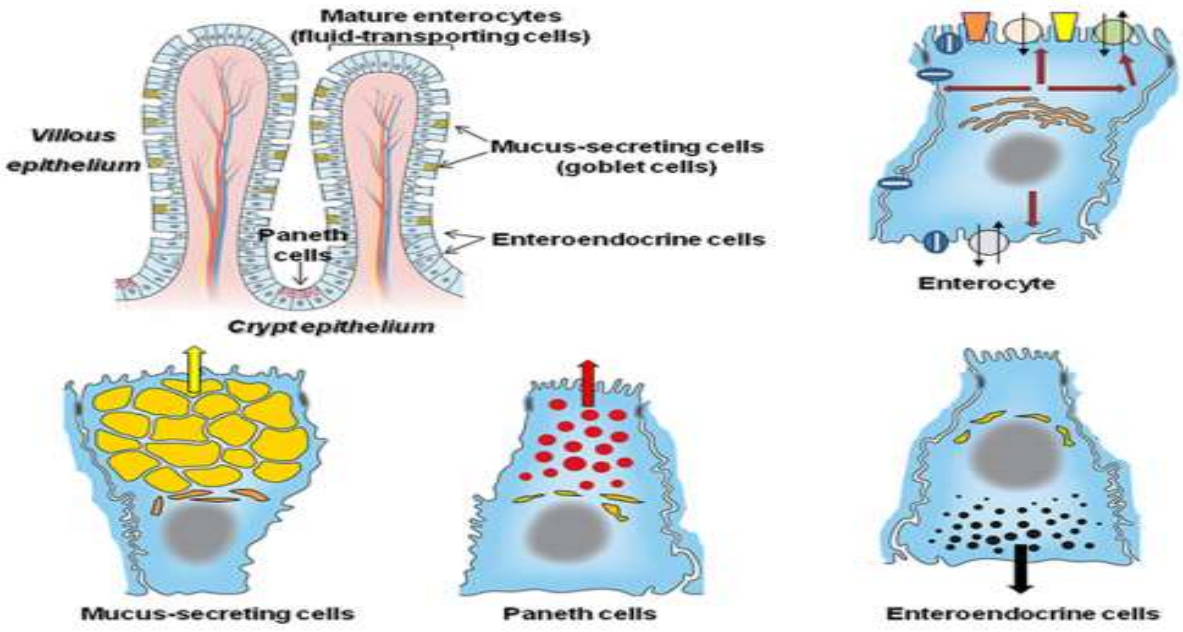


Figure 10. Schematic representation of intestinal epithelium barrier (Moal and Servin, 2013)

Disruption of the intestinal epithelial barrier associated with intestinal diseases is often caused by the deterioration of normal regulatory mechanisms that control gene expression, tight junction structure, and cytoskeletal signaling. Disruption of epithelial barrier integrity gives rise to several gastrointestinal diseases, including infection by pathogens, obesity and diabetes, necrotizing enterocolitis, irritable bowel syndrome and inflammatory bowel disease (Bron *et al.*, 2017). In addition to these, it might give rise to allergic diseases (Muñoz-quezada and Gil, 2012).

- *S. boulardii* preserves tight junction functions

The tight junction is a component of the apical junctional complex, that seals the paracellular space between epithelial cells. It is composed of transmembrane proteins, cytoplasmic adaptors, and the actin cytoskeleton. *S. boulardii* maintains tight junction by exerting a multifactorial effect which includes the inhibition of pro-inflammatory cytokines, such as IL-8, and preventing the activation of MAP kinases Erk1/2 and JNK/SAPK (Wang *et al.*, 2004). Furthermore, Bioactive factors released by *S. boulardii* trigger activation of various cell signaling pathways that give rise to strengthening of tight junctions and the barrier function. Related genes include *STE11, STE7, FUS3, KSS1, SSK2/22, PBS2, HOG1, BCK1,* and *SMK1*.

1.4.2. Mucosal Action

1.4.2.1. The Functions of the Mucus Layer

The mucus layer includes an inner and an outer layer. Inner mucus protects the apical epithelium whereas the outer mucus layer includes a large number of bacteria.

SCFA production

Short chain fatty acids (SCFAs) are known as volatile fatty acids produced by the gut microbiota in the large bowel as bacterial fermentation products from food components that are unabsorbed/undigested in the small intestine (Ríos-covián *et al.*, 2016). SCFA production in the colon is dependent on how rapidly carbohydrates are fermented. They are related to colonic absorption of water and electrolytes which effects the controlling of AAD.

S. boulardii enhances the normal level of SCFAs, producing acetic acid (C2), propionic acid (C3) and butyric acid (C4), important metabolites produced by the anaerobic flora, representing 90–95% of the SCFA present in the colon. Acetic acid is found mostly in the colon and makes up more than half of the total SCFA detected in feces.

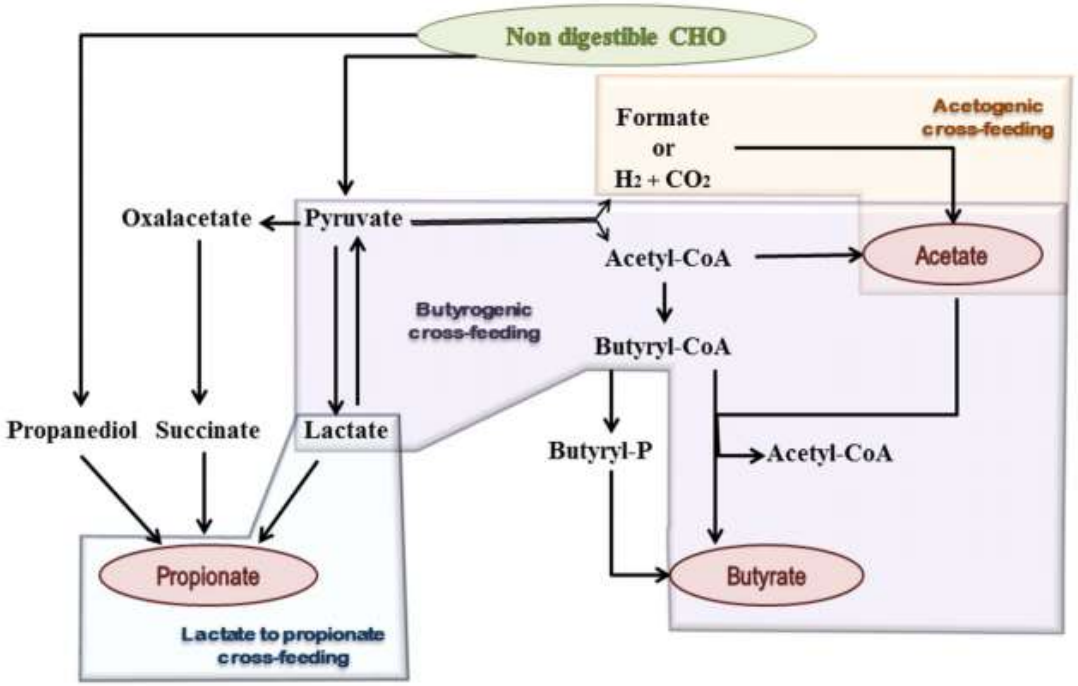


Figure 11.Schematic representation of SCFA formation microbial metabolic pathways in the human gut (Ríos-covián *et al.*, 2016).

Butyrate is the main energy source for intestinal epithelial cells (Figure 11). It influences epithelial cell proliferation, cell differentiation, mucus secretion, and barrier function in the large intestine. It has anti-inflammatory and antioxidant potential (Patel and Dupont, 2015). In addition, it inhibits NfkB activation (Distrutti *et al.*, 2016). Butyrate decreases bacterial translocation and improves the organization of tight junctions. It induces the production of mucin, a glycoprotein preserving the integrity of the intestinal epithelium.

Epithelial goblet cells secrete mucins. Mucins can be found bound to the brush border membrane or packaged within large intracellular vesicles, as shown in Figure 10 as yellow vesicles, that upon exocytosis into the luminal compartment form a thick mucus layer overlying the epithelium. Butyrate specifically regulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose. Recent studies have demonstrated that mucin secretion is promoted by SCFA produced during carbohydrate fermentation. Hence, the metabolism of butyrate in colonocytes is closely linked to some of its gene-regulating effects (Gaudier *et al.*, 2004). Interestingly, *Sb* has been indicated to induce the secretion of mucins and defensins from the host (Vandenplas, Brunser, and Szajewska 2009). A clinical study also has shown that increase fecal SCFA concentrations especially butyrate concentration in patients. They hypothesized that increase of fecal SCFA concentrations especially butyrate, *S. boulardii* may have preventive the effects of treating on TEN-induced diarrhea (Schneider et al. 2005).

In addition, fatty acids or their monoglyceride derivatives have long been known as antimicrobial agents that kill Gram-positive and Gram-negative bacteria. They also show antiviral and antifungal activity.

Prevention of microbial pathogen adherence

Recent studies have suggested that *S. boulardii* has a protective effect against *Escherichia coli, Vibrio cholerae, Salmonella sp., Candida albicans, C. difficile* and *H. pylori* infections (Khatri *et al.*, 2017; Kareem *et al.*, 2018). Two of the ways in which *S. boulardii* is predicted to exert its anti-bacterial activity is through the production of adhesion proteins and through the inhibition of pro-inflammatory cytokine production (Figure 12).

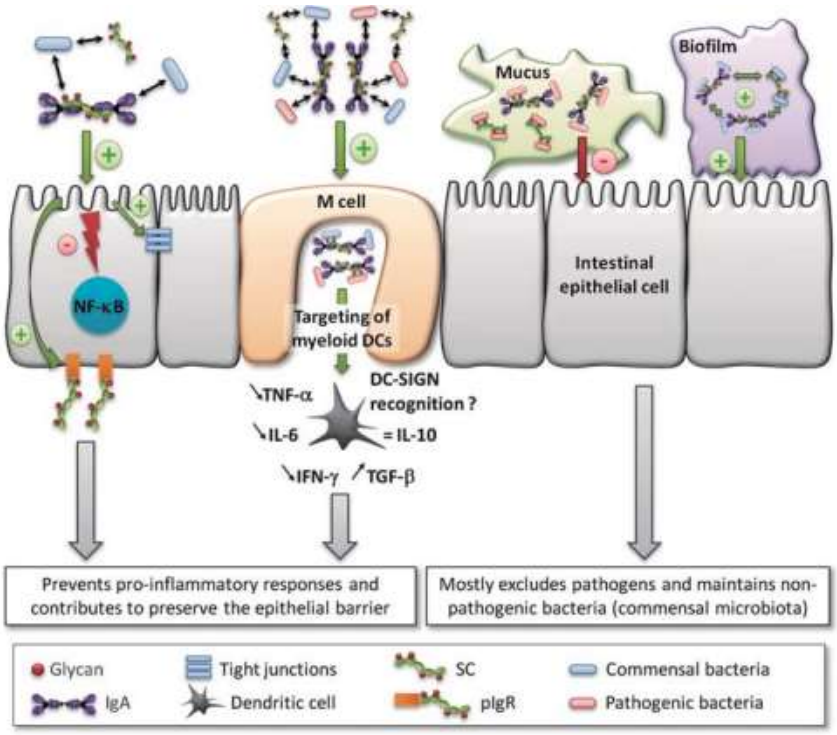


Figure 12. Bacterial adhesion and biofilm formation (Mantis *et al.*, 2011)

Adhesion & Flocculation

Adhesion to intestinal mucosa plays a key role for colonization and is important for the interaction between *S. boulardii* and the host, modulation of the immune system and antagonism against pathogens (Muñoz-quezada and Gil, 2012).

S. boulardii acts by producing adhesion proteins, such as flocculins, that favor the adhesion of *S. boulardii* cells to pathogenic bacteria cells, thus inhibiting their interaction with intestinal receptors and subsequent host invasion (Khatri *et al.,* 2017; Tiago *et al.,* 2012).

Flocculation genes in *S. boulardii* include *FLO1*, *FLO5*, *FLO8*, *FLO9*, *FLO10*, *FLO11*, *FIG2, EFG1* and *AGA1* (Khatri *et al.*, 2017).

1.4.2.2. Antimicrobial Effects

63-kDa Phosphatase LPS

EHEC infection leads to inflammation and disruption of the epithelial barrier. Dahan et al. (2003) showed that EHEC infection induced TNF-α synthesis that is implicated in apoptosis of T84 cells. *S. boulardii* was found to stimulate a decrease in TNF-α and related apoptosis in EHEC-infected T84 cells. *S. boulardii* blocks nuclear factor (NF)-κB activation, IL-8 gene expression, IL-8 production, TNF-α gene expression and secretion by lymphoid and non-

lymphoid cells. This effect has been linked to the activity of a 63-kDa *S. boulardii* protein phosphatase, that inhibits the toxicity of *E. coli* surface endotoxins (Buts *et al.*, 2006) This phosphatase may be encoded by the *PHO8, PRP3, JIP4, SNF1, SNM1, PEX29, CWC21, VPS52, VPS72, VP60, RIB3* or *PAC11* genes (Khatri *et al.*, 2017). *S. boulardii* displays high dephosphorylation activity (Buts and De Keyser, 2006) and was observed to decrease the inflammatory profile of LPS-activated dendritic cells and to block T-cell proliferation (Thomas *et al.*, 2009). Moreover, *S. boulardii* CNCM I-745 was found to secrete an alkaline phosphatase, with the capability of inactivating *Escherichia coli* lipopolysaccharide by dephosphorylation (Moré and Vandenplas, 2018).

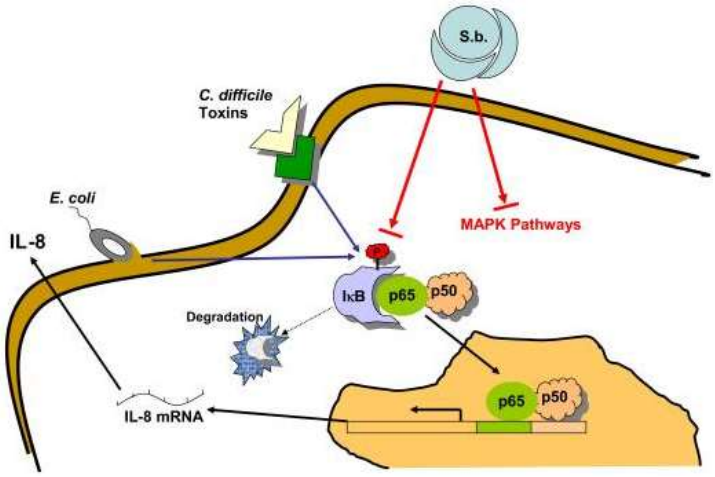


Figure 13.The mode of action *S. boulardii* according to bacterial infections (Pothoulakis, 2009)

54 kDa Serine Protease

S. boulardii expresses a 54-kDa serine protease that exhibits the ability to degrade *C. difficile* toxin A (Figure 13). One study showed that *S. boulardii* significantly decreases the liquid secretion and permeability caused by toxin A in rat ileum. Secreted serine protease by *S. boulardii* decreases the ability of toxins A and B to bind human brush border membrane and inhibits the pathogenic impact of both toxins on colonic epithelial cells (Dorota Czerucka and Rampal 2002 ;Vandenplas, Brunser, and Szajewska 2009). *S. boulardii* might additionally behave in the intestinal lumen by blocking the toxin receptor (Pontier-bres *et al.*, 2014; Graff *et al.*, 2008).

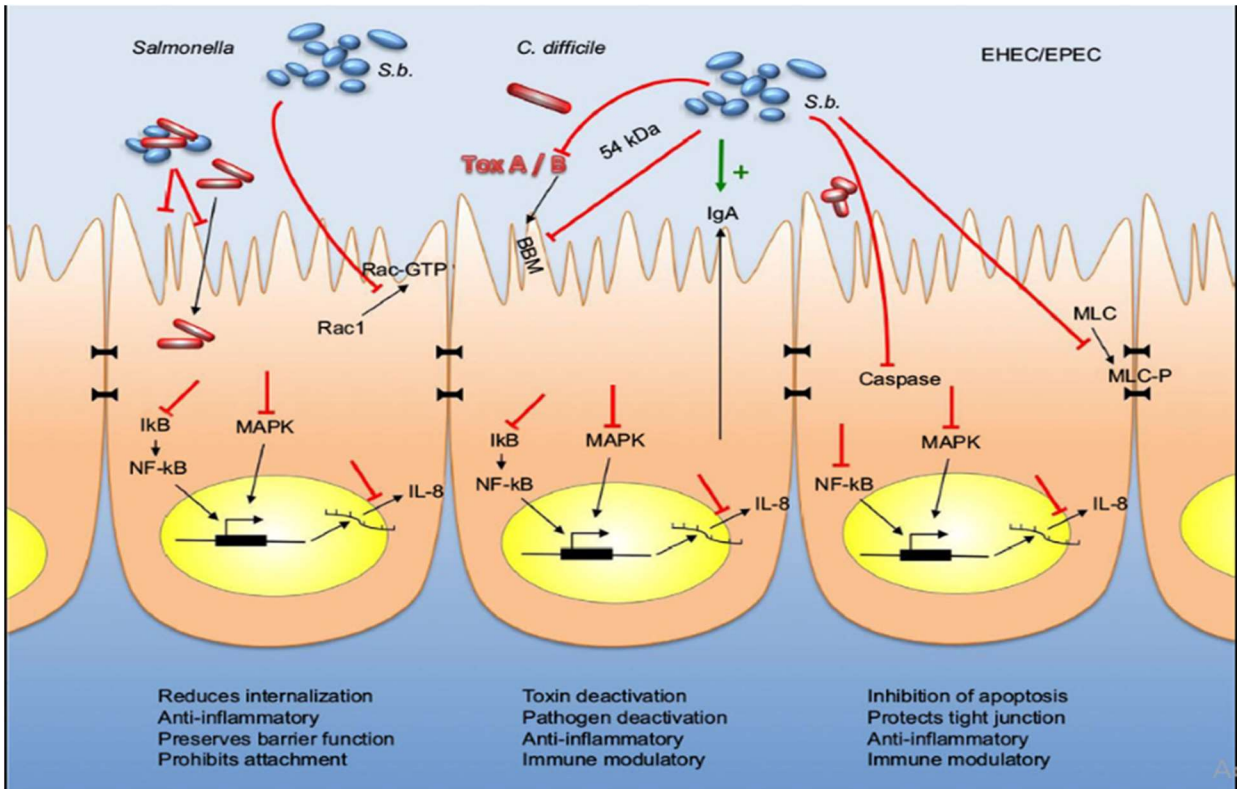


Figure 14. Mode of Action of *S. boulardii* against *E. coli, C. difficile* and *Salmonella* infections (Stier H., 2016)

120-kDa protein

In vivo studies show that *S. boulardii* administration leads to inhibition of *Vibrio cholerae* or enterotoxic *Escherichia coli* (ETEC) pathogenesis. This effect was linked to diminished sodium and water secretion in intestinal loops, as well as decreased *V. cholerae* toxin-induced cAMP levels in rat intestinal cells (Kindenplas, 1999; Khatri *et al.*, 2017; Pontier-bres *et al.*, 2015). This protective effect was found to be related to a 120 kDa protein (Czerucka, Roux and Rampal, 1994) (Figure 14).

120 kDa secreted proteins in *S. boulardii* are encoded by *KIN1, MAD1, TFC4, VAS1, KAP120, PIK1, NMD5, JSN1, PUF2, RGC1, ENA5,KCS1, SEG2, NUP120* and *MSH3* genes (Khatri *et al.*, 2013).

1.4.2.3. Toxin related signaling

cAMP is known as a potent second messenger that is associated with various stimulatory processes in many cell types. Cholera toxin is an enterotoxin that stimulates cells by increasing the synthesis cAMP. Vasoactive intestinal polypeptide (VIP) and prostaglandins (PGE_2), mediators in cholera pathogenesis, recognize receptors coupled to adenylate cyclase. These

mediators induce Cl^- secretion by a cAMP-dependent signal transduction pathway. Apparently, *S. boulardii* may interfere with the adenylate cyclase-cAMP transduction pathway and Cl^- secretion. In addition, Dorota Czerucka and Rampal (1999) determined that *Sb*, through the activity of a 120 kDa protein, exerts inhibitory influence on Cholera-induced cAMP concentration an $^{125}I^-$ efflux in T84 monolayers, inhibits receptor-mediated and non receptor-mediated cAMP-induced secretion, reduces $^{125}I^-$ efflux but not 1,4,5-triphosphate in carbachol-treated cells, and stimulates 1,4,5-triphosphate synthesis in T84 cells.

S. boulardii decreases the activation of Erk1/2 mitogen-activated protein kinase and, consequently, IL-8 secretion induced by *C. difficile* toxin A. *Sb* further reverses the drop in intestinal permeability after exposure to *C. difficile* toxins A and B (Pontier-bres *et al.*, 2015).

An animal study showed that *S. boulardii* decreases the levels of inflammatory cytokines and activates mitogen-activated protein kinases (p38, JNK, and ERK1/2), phospho-IkB, p65-RelA, phospho-jun and c-fos in the colon, belonging to signaling pathways involved in the activation of inflammation stimulated by *Salmonella typhimurium* (ST). When ST binds to *Sb*, this action reduces ST translocation which results in diminished activation of inflammation-associated and signaling pathways, leading to reduced intestinal inflammation in a murine model of typhoid fever (Martins *et al.,* 2013).

1.4.3. Immunomodulation effect

Increased secretion of immunoglobulin SIgA levels

Lamina propria is defined as the connective tissue that underlies the epithelium of the mucosa and contains various myeloid and lymphoid cells, including macrophages, dendritic cells, T cells, and B cells. Dendritic cells (DC) are involved in the control of T cell activation, by inducing the activation of naive T cells (Thomas *et al.*, 2009).

Recent studies have reported that when *S. boulardii* attaches to dendritic cells (DCs) and increases the secretion of immunoglobulins A and M and of cytokines, including interleukin (IL)1β, IL-12, IL-6, TNF-α and IL-10 (Stier H., 2016). Secretory IgA (SIgA) is the first line of defense against enteric toxins and pathogenic microorganisms (Mantis *et al.,* 2011). *S. boulardii* exposure leads to increased intestinal secretion of immunoglobulins, including immunoglobulin A (IgA), leading to improved defence against pathogens. But and colleagues observed on rat small intestine that *S. boulardii* dramatically enhances the secretion of immunoglobulin A (IgA)

(Buts *et al.,* 1990). When *S. boulardii* leads to increased IgA secretion, it enhances the immunologic barrier produced by the intestinal mucosa (Peniche, Savidge, and Dann 2013; Seekatz and Young 2014).

1.4.4. Trophic Action

When *S. boulardii* is orally administrated, it upgrades intestinal functions by three important mechanisms which are:

- Endoluminal secretion of substantial amounts of spermine and spermidine which after absorption increase the intracellular pool of polyamines and the synthesis of brush border membrane (BBM) glycoproteins, enzymes, and carriers;
- Endoluminal secretion of enzymes by the yeast cells itself;
- Activation of messengers which transduce mitogenic and metabolic signals from the apical membrane to the nucleus using the (di) phosphorylation of intracellular serine, threonine and tyrosine kinases.

Enzymatic activity:

S. boulardii secretes, during its intestinal transit, enzymes such as lactase, maltase α-glucosidase, sucrase-isomaltase, maltase-glucoamylase, α,α trehalase, a zinc-metalloprotease acting as a leucine aminopeptidase and alkaline phosphatase able to dephosphorylate bacterial endotoxins (Border *et al.*, 2010). *S. boulardii* stimulates the expression of disaccharidases, sucrases, production of glycoproteins in the microvilli.

One *in vivo* study showed that *S. boulardii*, when administered orally to rats, upgrades endoluminal N-terminal hydrolysis of oligopeptides allowing aminopeptidase to move within the lumen. This action could be important on inhibiting reactions to food antigens, while mucosal permeability is enhanced (Buts *et al.*, 2002).

Polyamines secreted by *S. boulardii* also induce protein synthesis via RNA binding and stabilization, resulting in an increase in growth-related and differentiation-related proteins, including digestive enzymes such as lactase, maltase, sucrase, which will be inserted into the brush border membrane (Vandenplas, Brunser, and Szajewska 2009; Moré and Vandenplas 2018). Polyamines further protect lipids from oxidation and increase the activity of short-chain fatty acids (SCFA) (Kareem *et al.*, 2018).

Table 3. Genes predicted to underlie *S. boulardii* mode of action as a probiotic agent.

***S. boulardii* probiotic effect**	**Related disease**	**Related Pathways**	***Sb* mode of action**	**Genes underlying *Sb* mode of action**	**Reference**
Anti-toxin effect	Diarrhea and colitis caused by *C.difficile* infection	x	Production of a 54 kDa serine protease, protecting against toxin A and B	(Serine protease) *PRC1* *GLN3* *GAT3* *RRT12* *YSP3*	(Khatri et al. 2017, 2013 ; Pothoulakis, 1993; Castagliuolo, Mont, and Nikulasson 1996;)
Anti-toxin effect	Diarrhea caused by *E. coli*	NF-κB and Mitogen-Activated Protein Kinase (MAPK) signaling pathways	Secretion of a 63 kDa protein phosphatase which inhibits *E. coli* toxins	(Phosphatase) *PHO8* *PRP3* *JIP4* *SNF1* *SNM1* *PEX29* *CWC21* *VPS52* *VPS72* *RIB3* *PAC11*	(Khatri *et al.*, 2013, 2017)
Anti-toxin effect	Diarrhea caused by *Vibrio cholera*	cAMP pathway	Secretion of a 120 kDa protein that neutralizes cholera toxin by decreasing cAMP levels in the intestinal cells	*KIN1* *MAD1* *TFC4* *VAS1* *KAP120* *PIK1* *NMD5*	(Khatri *et al.*, 2013)

				JSN1 *PUF2* *RGC1* *ENA5* *KCS1* *SEG2* *NUP120* *MSH3*	
Anti-microbial effect	Irritable Bowel Diseases (IBD)	Butyrate metabolism	SCFA production, especially butyrate	ACC1 HFA1	(Klug, 2014)
Anti-microbial effect	AAD related to pathogen infection	Protein secretory pathway	Increased adhesion protein production	*FLO5* *FLO8* *FLO9* *FLO10* *FLO11* *FIG2* *EFG1* *AGA2(SAG1)*	(Khatri *et al.*, 2017)
Trophic Effect	Lactose intolerance	Galactose metabolism & Leloir pathway	Lactase overexpression for degrading lactose	*MIG1* *PGM1* *GAL7* *GAL10* *GAL1* *CYC8* *GAL2* *GAL4* *GAL80* *PGM2* *GAL3* *TUP1*	(Khatri *et al.*, 2017)

Trophic Effect	Obesity Type 2 diabetes	Phosphatidate biosynthesis I (dihydroxyacetone pathway)	Activation of lipid degradation in dendritic (DCs) cell	*TGL2/3/4/5* *AYR1* *TGL1* *YJU3* *YPC1* *YDC1*	(Klug, 2014)
Prevention of tight junction distribution	Irritable bowel syndrome (IBS), gluten intolerance, gastroenteritis, and *H. pylori* infections	MAP kinases Erk1/2 and JNK/SAPK.	Inhibition of MAP kinases Erk1/2 and JNK/SAPK	*STE11* *STE7* *FUS3* *KSS1* *SSK2* *PBS2* *HOG1* *BCK1* *SMK1*	(Schaeffer and Weber, 1999)
Increased immune defense in the gut	Allergic diseases	Arginine and proline metabolism	Polyamines secretion	*SPE2* *SPE3* *CAR1* *CAR2* *PUT2* *PUT1* *PRO1* *PRO2* *PRO3*	(Khatri *et al.*, 2013)
Immunomodulation effect	Gluten intolerance & Celiac Disease	Palatinose metabolism	Increased production of immunoglobulin IgA by epithelial cells Upregulation of palatinose uptake and metabolism	(Isomaltase) *IMA1*	(Khatri *et al.,* 2017)

1.5. Differences between *S. boulardii* and *S. cerevisiae*

S. boulardii survives transit through the GI tract both *in vitro* and *in vivo* and inhibits the growth of a number of microbial pathogens. Indeed, *S. boulardii* can live longer in the gut than *S. cerevisiae* (Łukaszewicz, 2012; Liu *et al.*, 2016). In this context, it is interesting to observe that while *S. cerevisiae* strains grow and metabolize at an optimal temperature of 30ºC, *S. boulardii* grows optimally at human body temperature, 37ºC. Additionally, *S. boulardii* grows more rapidly than *S. cerevisiae* (Fietto *et al.*, 2004) and is more tolerant to low pH and bile acids. Possibly due to these characteristics, *S. boulardii* has been shown to be more resistant than *S. cerevisiae* to gastric conditions (Fietto *et al.*, 2004). The gastric environment has extremely low pH which is generally ~2.0. At this pH, *S. boulardii* proteins continue to be positively charged, thus remaining able to establish electrostatic interactions with negatively charged components of the cell wall of gut bacteria, a requirement for its probiotic activity (Urdaci, 2008).

There are main discriminatory metabolites between *Sb* and Sc which are trehalose, myo-inositol, lactic acid, fumaric acid and glycerol 3-phosphate (Łukaszewicz, 2012). Mackenzie et al. (2008) determined that non-medical *Sc* strains have the capability of producing lactic acid, valine, fumaric acid, malic acid, glycerol-3-phosphate and TCA cycle intermediates such as fumaric, citric, isocitric, succinic and malic acids. On the other hand, 4-Hydroxyphenylethanol related to tyrosine metabolism, 2,3,4-Trihydroxybutanal, Pentonic acid 1,4-lactone, myo-inositol are synthesized by *S. boulardii* (Mackenzie *et al.*, 2008).

Despite the observed phenotypic differences, a study focused on the analysis of the genome sequences of five *S. boulardii* strains used commercially as probiotics, has shown that the genome of *S. boulardii* is 99% similar to that of *S. cerevisiae* (Edwards-Ingram *et al.*, 2007; Khatri *et al.*, 2017). The surprising observation that *S. cerevisiae* and *S. boulardii* are very similar in terms of their genomic sequence, raises the question of what are the features that make *S. boulardii* a probiotic, while *S. cerevisiae* is not.

1.6. Objectives and work outline

The hypothesis that this MSc project explores is that the difference in probiotic activity observed in *S. boulardii* when compared with *S. cerevisiae* may rely on differences at the level of transcription regulatory control of probiotic activity-related genes. To evaluate that in a systematic way, an analysis of transcription regulation in *S. boulardii* is required, which prompted us to start building the ProBioYeastract database and develop additional strain-comparison tools. The ProBioYeastract database was constructed using the structure developed for the YEASTRACT database and this MSc project contributed to its development.

The YEASTRACT (Yeast Search for Transcriptional Regulators And Consensus Tracking) database is a repository of curated published transcriptional associations, that offers tools for transcription regulation analysis in yeast. In its first release, it focused solely on the model yeast *S. cerevisiae* (Teixeira *et al.*, 2006; Teixeira *et al.,* 2018) but more recently, it was expanded to pathogenic yeasts of the *Candida* genus in the form of the PathoYeastract database (Monteiro *et al.*, 2017)

The first chapter of the MSc thesis is an Introduction to current knowledge on the mechanisms of *S. boulardii* probiotic activity, especially in comparison to non-probiotic strains of *S. cerevisiae*. In the second chapter, the used *in silico* and wet-lab methods are described. The third chapter describes and discusses the obtained results, starting from the *in silico* analysis of the predicted regulators of probiotic-related genes, in the search for using ProBioYeastract and YEASTRACT databases. Among the genes whose expression appears to be controlled differently in *Sb* strains, when compared to S288C, 6 were selected for gene expression measurement, through RT-PCR, to evaluate the *in silico* analysis results. Based on the gene expression results, the adhesion, aggregation and biofilm formation of *S. boulardii* CNCM I-745 (ULTRA-LEVURE®) was examined, in comparison to *S. cerevisiae BY4741*. The forth chapters presents final Conclusion and Future perspectives, focusing on the most significant aspects of our results, and in the still unanswered questions on the mechanism probiotic activity and gene regulation in *Sb*.

2. METHODOLOGY

2.1. Cross-strain promoter analysis: *Sc* vs *Sb*

In the beginning of the cross-strain promoter analysis, the data used for the construction of ProBioYeastract considered the following assemblies provided by GenBank for Unique 28 (https://www.ncbi.nlm.nih.gov/genome/16045?genome_assembly_id=256035) and Biocodex (https://www.ncbi.nlm.nih.gov/genome/16045?genome_assembly_id=256034). Using scripts, these assemblies were parsed and the data was loaded to the ProBioYeastract database. The information of orthology between *Sc* and *Sb* was provided by the annotation already in the assembly, meaning that the ones submitting the genome, did a functional analysis, and for each gene, they obtained the best hit against *Sc* genes and annotated these as being orthologous genes.

Afterwards, *S. boulardii* Unique28 and Biocodex gene promoters were retrieved from the ProBioYeastract database (http://146.193.39.124/ ProBioYeastract/sboulardii/index.php) and *S. cerevisiae* gene promoters were retrieved from the YEASTRACT database. After that, the existence of *S. cerevisiae* putative transcription factor binding sites in the promoters of the three strains were compared by using the YEASTRACT database (http://www.yeastract.com/) query "Find TF binding site".

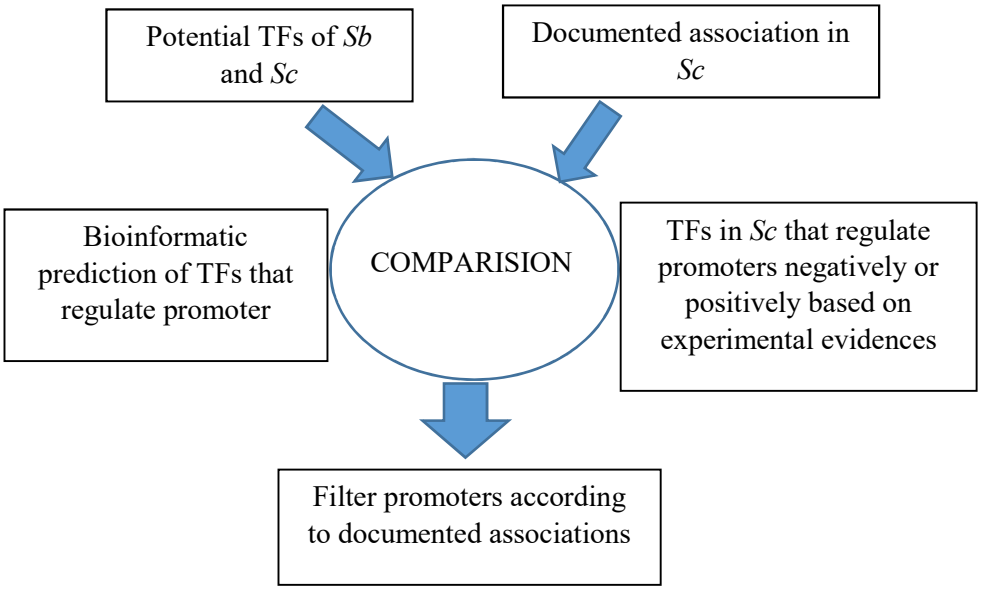


Figure 15. The pipeline of cross-strain promoter comparison using YEASTRACT bioinformatics tools.

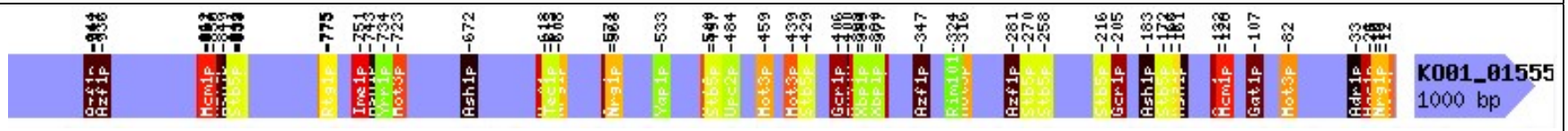
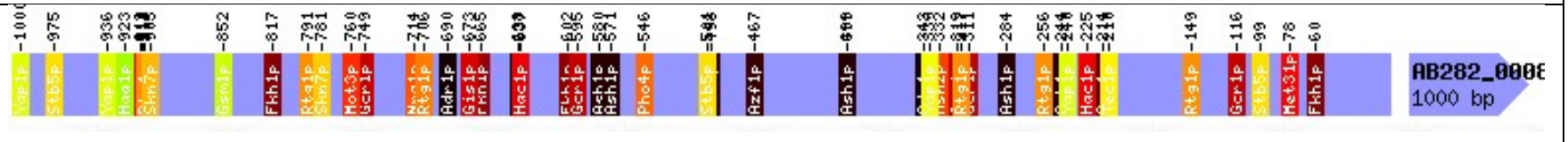
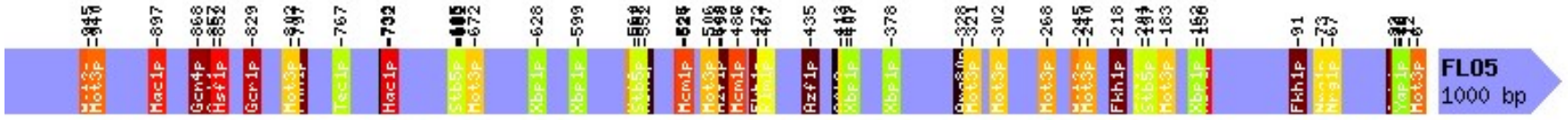
As an example, the analysis of the *FLO5* gene (encoding a lectin-like cell wall protein (flocculin) involved in flocculation) is detailed below.

This process was repeated for each predicted probiotic-related genes, compiled from the literature (Table 3). The pipeline design was used as the basis for the development of the "Cross-strain comparison" tool that is now present in the ProBioYeastract database (http://146.193.39.124/probioyeastract/sboulardii/formcrossstrain.php).

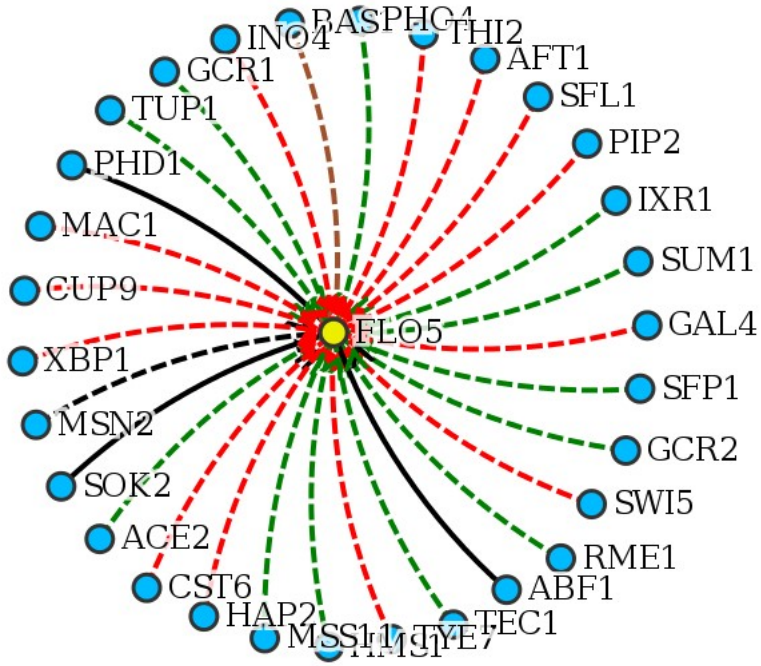
The promoters of the *FLO5* genes from Biocodex, Unique28 and S288C strains were extracted (Table 4) and compared in terms of presence or absence of *S. cerevisiae* TFBS (Table 4). Afterwards, the results were filtered to consider only the TFs that are known to regulate the *S. cerevisiae* S288C *FLO5*, obtained by using the YEASTRACT "Find TF" query.

Table 4. An example of a cross-strains promoter analysis, using ProBioYeastract and YEASTRACT database

Promotor Sequence extraction	
For *S. boulardii* Biocodex by using ProBioYeastract database	>KO01_01555 upstream sequence, from -1000 to -1, size 1000 AAAAAAATCCATTACATTACGTTCCTTGCTCTGGATCTCTTCACCTGCAATTACTGAAAAGAAAAAAAGAAATATAATT TTCCTTTGAGCAGCCTTGGTAACTTATCTAAGAACACGCGATGTAATCGAAATTTAATAACCTAAAAAAGAAATAGAGA TCGGTGACGTTTGACGCTAGCAAATCTCCACGAGATTTATCTGGAAGGGCATCACCCGTCAAAACACTAAGAAATTTGA CACCTTATTTTTTTTTCTCCGAAACGTTCTGAGACGTTTCTTTTTTTTTTCAGGACGCCACCCTAAATCCGAAAAACCTTAC TTGGCATTAGTGTTGTAAAAATTTCTTTTTTCCATCGTGGTTCTGAAAAGCTTGCAGAAAAAAAGTTCGAGAACGTTTTC GTATGACTTATTAAGCTGCCCTTGTACTTGCCTTCCCTCTTCTTTACTATTTTCAGCCAGGGACAGAAATAAAGCAAAAT CGTTTAACTTTTATGACCGTAACAGGTAACAATATCTGCTCAATTCGGTTAGTAAAAAAAAAAAAGGTTATCGTAGGTT TACTATTTTCACATCGGTGCATAGAATCTTTACTCTACTTCCTTTGGAGGGAATTTAGTAGTGCTCGATTAGCAGATAAC AGCGCAGGGTTCTATGTAGTACGAAGCACTATCAGGTTTTTTAACGTACGTGAGTTGTTTTTTGTTTCTTATTTTTGGCTT GCCTGGGATAAACGGTGCCCATCAAATTATTAAGACCTGGTGAAAAGAGCGTACCTACATGGGTATCGGGGTTTTTGCC AGAAAGAGCTTTAACTGCAATTGATGCCTACCGGAGATTATTTTGATATCAAAATGCAGGAACGATATCTACTTCCCTA AAGCAGAAAACAAAACATTTTTTTAAAAGTCTTTCTTATATAAAGGTAGCCTTCATTTCATGATAGCCAGATGATAATC TTAATGTAACAATTGGAGGATACCAGCATCCCTCCACACCTACAA
For *S. boulardii* Unique28 by using ProBioYeastract database	>AB282_00080 upstream sequence, from -1000 to -1, size 1000 TGACAATGCCTCATCGCTATATGTTTTTGGCAGTCTTTACACTTCTGGCACTAATTAATGTGGCCTCAGGAGCCACAGAG GCGTGCTTACCAGCAGGCCAGAGGAAAAGTGGGATGAATATAAATTTTTACCAGTATTCATTGAAAGATTCCTCCACGT ATTCTAATGCAGCATATATGGCTTACCAATATGCAGACAAAGTCAAATTGGGCTCTGTTAGTGGGCAAACGGATATATC TATCAACTATAATCTTCCTTGTGTTACAACCTCAGGGACATATCAGTGCCCTCAAGAAGATGCATATGGTAATTGGGGA TGCAGAGGTAAGGGGAGATGCTCCAACAGTCAAGCAGTTTCATACTGGAGTACAGATCTGTTTGGCTTTTATACCACTC CAACAAACATCACCCTAGAAATGACAGGTTACTTTTTACCACCACAGACAGGTTCTTACACGTTTTCTTTTGCAACAAT AGATGATTCTGCAATTTTATCAGTCGGTGGTAGCATTGCGTTCGAATGTTGTGCACAAGAACAACCTCCCATCACATCG ACTAACTTCACCATCAATGGTATCAAGCCATGGCATGGAAGTCTCCCTGATAATATCGCAGGGACTGTCTACATGTATG CTGGTTTCTATTATCCAATGAAGATTGTTTACTCAAATGCCGTTTCCTGGGGTACACTTCCAATTAGTGTGACACTACCA GATGGCACTACCGTTAGTGATGACTTTGAAGGGTACGTATATACCTTTGACAACAATCTAAGCCAGCCAAACTGTACCA TTCCAGACCCTTCAAATTATACTGTCAGTACTACCATAACTACAACCGAGCCATGGACCGGTACTTTCACCTCTACGTCT ACTGAGATGACTACTATCACTGGCACCAACGGTGTACCAACTGACGAAACCATCATTGTTGTCAAAACACCAACAACT GCTAGCACCATCATAACTACGACCGAAGCATGGACTGGCACTTTCACATC

S. cerevisiae S288C by using YEASTRACT Database	>FLO5 6322005 upstream sequence, from -1000 to -1, size 1000 CCTCTTTCTTTTTTGTAAAAAATTCTGTTTTTAATAGCCAGTTCTTTAGTGATTACAGGTAAGAGGGTTTCATATTTTAGA AGTGCAGCCATGATGAAGCACTTTTGCTCATTTATTGCGAGAAGTTTAATAAGTAGTATGGTTCCATTTTCAAGAATCG AGGCACTGTTCCTTCCCAACCTTGGAATCATACTCCGAAAGGATTTCAAGCCGATTTAAATTCACCTGGTAACTTTCCTA CGGTTTGGCCCAAGGTGATTATAATTAACTTGCGGCTTGTTTTCAGCCTGCGATCGAACCTTTTTTACGCAAAAAAACCT TATTAATTAAGGTTTTGAAAATTTTCTTCTTTCCGGGAGATTTTCATGTAGCCTCGAGCTTCTGGATTCTCACGGGATTAT CTCGCGTTACATTTTTTACTTTCTTCTTTCTTTTTGACTTAGGATATACAGATGATACGTCATTGTGTCATAAAACCCGCT GTTGTGCAACAAAAGGGAAAAAGAAAAATACTCCTTTTTAGGTCTTATAAATATTTTTAGCAGCCATCAAGTCCGGCTT TCAAACTTAATTTCACCCTTTTTCACGGCACCCTCGAGAATTACACTTTGGTTGCATGCAGGAGTACGCGAAATGCAGC ATAAGCTACACATCTATGCGTAGATCGCTTAACCTCTAAAGGCCGTAAACTTTTATTTTGTTTTGCGCTCATTAAAACCT AGTGGGAGCTGGTAGGAAATAAGCTAGTAGCTTCTATGGATAGAATGGAAATAAACGTAGGTGTAAACACTATTGGTA GAGAAGTTCCTCTGGTCAAATTTTCATGGGAGATACGTTAAATCTTTCACAGTCTTATCGTTTTGAATCACTGGACGGTT CTGGTATTCTGCTTCATATTTCGACAAGATAATAAATATAAAAAGAGCACCCTCATGATTTCTTGCTCTGCAGTAAATTC CGCAAATGATTTTCTTTAAATTGATTAGCACCACTAAAAAAA
Determination of TFs Binding Sites Location	
For Biocodex *FLO5*	
For Unique28 *FLO5*	
For *S. cerevisiae* S288C *FLO5*	

Determination of documented *S. cerevisiae* TFs that regulate *FLO5*



TFs for which there is at least one TFBS in the *FLO5* gene promoters, known to regulate *S. cerevisiae FLO5* expression

Biocodex (*S. boulardii*)	Unique 28 (*S. boulardii*)	*S. cerevisiae*
x	x	Aft1p

TFs for which there are unique TFBS in the *FLO5* gene promoters

Unique to *Sc*	Aft2p, Aft1p, Arg80p, Gcn4p, Mac1p, Rlm1p, Sfl1p
Unique to Biocodex	Cst6p, Ime1p, Sko1p, Upc2p, Cad1p, Yap3p, Cin5p, Yap5p, Yrr1p, Rim101p
Unique to Unique28	Haa1p, Gsm1p, Skn7p, Pho4p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Crz1p

Unique to *Sc* and active in *Sc*	Aft1p
Unique in both Biocodex and Unique28	x

As a result of this *in silico* analysis, 26 out of the 83 probiotic-related genes were found to have TFBS that appeared uniquely in the *S. boulardii* promoters. Among these 6 were chosen for experimental evaluation, representative of the probiotic effects attributed to *S. boulardii* (Table 5).

Table 5. Summary of selected genes for experimental evaluation after cross-strain promotor analysis

Related Disease	Pathways	Selected genes for experimental trials
AAD related to pathogen infection (Adhesion)	Protein secretory	*FLO5*
Candidiasis (Adhesion)	cAMP-PKA	*EFG1*
Obesity, Type II diabetes	Phosphatidate biosynthesis I (dihydroxyacetone pathway)	*TGL4, YDC1*
Allergic Diseases (Polyamine secretion)	Arginine and proline metabolism	*SPE2*
Gluten Intolerances	Palatinose metabolism	*IMA1*

2.2. Gene Expression Analysis

To assess the expression of the selected genes in *Saccharomyces boulardii* and *Saccharomyces cerevisiae* strains, three steps were taken, as illustrated in Figure 16: cell cultures to retrieve biomass; RNA extraction and RT-PCR, to measure relative gene expression.

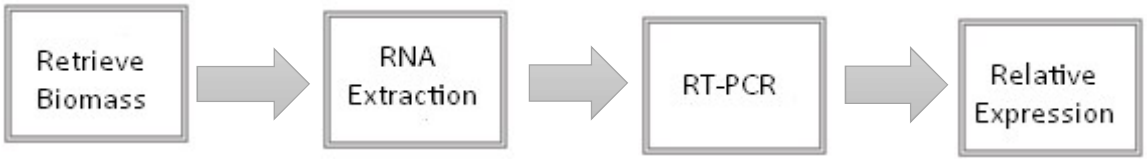


Figure 16. Scheme of experimental methodology to determine the expression of the selected genes.

2.2.1. Yeast strains and Growth Conditions

Saccharomyces boulardii CNCM I-745 was isolated from an ULTRA-LEVURE® (Biocodex, Beauvais, France) sachet in YPD solid agar. *Saccharomyces cerevisiae* BY4741 strain was obtained from Euroscarf collection.

A small amount of *Sc* and *Sb* strains were collected from solid media and transferred into YPD liquid medium (20g/L glucose (Merck), 10 g/L yeast extract, 20 g/L peptone) (25 ml) in an erlenmeyer flask. The culture was kept under agitation (250 rpm) at 30°C in YPD medium overnight. Cell growth was measured by assessing the optical density (OD) at 600 nm of the cell suspension, to determine the volume of culture to be transferred to a new flask with fresh YPD or YPD+cholate (including 0.5 g/l sodium cholate (Sigma), to mimic human gastrointestinal environmental conditions (Fietto *et al.*, 2004) medium in order to start with an OD600nm = 0.1. the new flasks were kept under agitation (250 rpm) at 30 °C for 5h to ensure 3 cell duplications, when an OD600nm of 0.8 was reached. Afterwards, cells were harvested by centrifugation at 7 000 rpm at 4 °C for 5 min. Prepared samples were stored at -80°C freezer until RNA extraction, as indicated Fig 17.

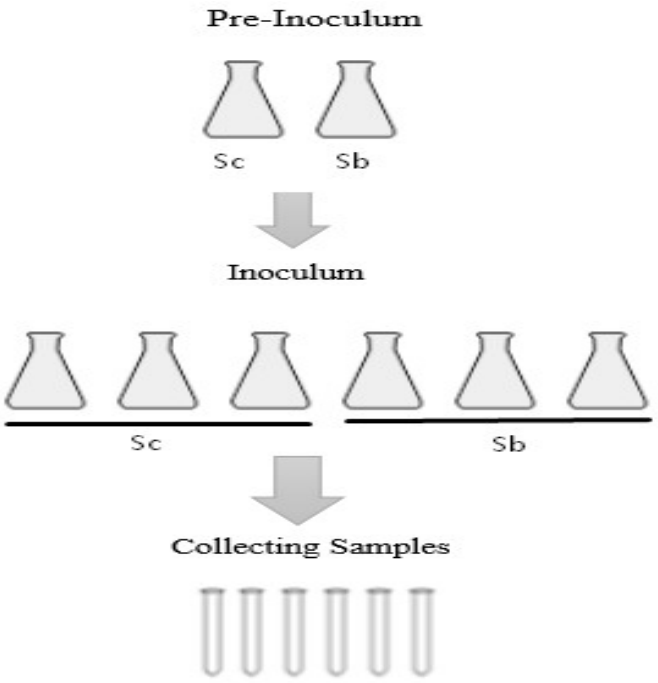


Figure 17. Scheme of experiments of the growth of cells carried out to RNA extraction

2.2.2. RNA Extraction

The total RNA extraction was carried out for three replicates of *S. boulardii* and *S. cerevisiae.* Firstly, The pellet of cells was resuspended in 900 µl of AE buffer (50 mM NaAc (Sigma), 10mM EDTA (Aldritch), pH=5.3; 0.1% (v/v) diethylpyrocarbonate (DEPC) treated). Then, 90 µl of SDS 10% were added and mixed by vortexing for 5 seconds. After that, 800 µl of phenol for RNA extraction was added and mixed by vortexing for 5 seconds. After adding phenol, the mix was incubated at 65ºC for 4 minutes. After incubation, the eppendorf tubes were kept on dry ice. Then, each mixture was centrifuged at 15000 rpm at 4ºC for 5 minutes, and the upper liquid phase transferred to a new Eppendorf. 400 µl phenol and 400 µl chloroform were then added and mixed by vortexing for about 5 seconds and centrifuged at 15000 rpm at 4ºC for 5 minutes. The upper liquid phase was transferred to a new Eppendorf and the previous step was subsequently repeated once again. Afterwards, 90 µl sodium acetate (Merck, 3M, pH=5.3, 0.1% DEPC - diethyl pyrocarbonate) and 1 mL 100% ethanol at -20 ºC were added to the collected supernatants, mixed by vortexing for 5s and then stored at -20ºC for 20 minutes, for RNA precipitation. The samples were then centrifuged at 15000 rpm, at 4ºC for 20 minutes, and the supernatant was discarded. Afterward, 750 µl 70% (v/v) ethanol was added and the samples were centrifuged at 15000 rpm, at 4ºC for 15 minutes. The supernatant was discarded carefully by using a syringe. The pellets were dried in the SpeedVac (V-AL, 20 min, 45ºC) and resuspended in 30 µl distilled H2O with 0.1% DEPC.

NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used to measure RNA concentration and quality. The concentration was then adjusted to 500 ng/µl for the real-time RT-PCR experiments.

2.2.3. Real-Time RT-PCR

The RT-PCR procedure consisted of two main steps. In the first step, reverse transcription was performed. The reverse transcription (RT) converts RNA into cDNA (complementary DNA), which is then used in the real-time PCR process. PCR reactions were prepared for each sample according to the values indicated in Table 6. The retrotranscription program used is described in Table 7.

Table 6. PCR reaction mixture components and their volumes

Component	Volume in µl (per sample)
10X Buffer (10x)	1.0
$MgCl_2$ (25mM)	2.2
dNTP's (2.5mM)	2.0
Random hexamers (50 µLM)	0.5
RNase Inhibitor (20 U/L)	0.2
MultiScribeTM reverse transcriptase (50 U/L)	0.25
ddH_2O DEPC treated	1.85
RNA sample (500 ng/µL)	2
Total	**10**

Table 7. Thermal cycling parameters for the first step of the real-time RT-PCR

Step	Time (min)	Temperature (ºC)
Incubation	10	25
Reverse Transcription	30	45
Reverse Transcriptase inactivation	5	95

In the second step, Real-Time PCR reactions were prepared for each sample according to the Table 8. SYBR® Green reagent was chosen as detection chemistry to perform relative quantification of gene expression. Real-Time PCR was run and analysed using its own software 7500 Systems SDS Software Applied Biosystems (Table 8).

Table 8. RT- PCR reaction components and their volumes for each sample

Component	Volume in µl (per sample)
SYBR®Green PCR Master Mix (2x)	12.5
Forward primer (4 pmol/µL)	2.5
Reverse primer (4 pmol/µL)	2.5
cDNA	2.5

ddH_2O	5.0
Total	**25**

The aim is to find the number of cycles (Ct) necessary to reach a given level of fluorescence above the noise threshold (Wong and Medrano, 2005). Hence, the signal level is registered in an amplification plot, from which Ct is estimated by the intersection between the exponential phase curve and threshold line. The normalization of the Ct values is performed using an internal control indicated in Equation 1 (Rao *et al.*, 2013).

$$\Delta Ct = (target) - Ct(control) \quad \text{..... Equation 1}$$

Then, each normalized value correspondent to each gene is compared with the physiological calibrator considered, as shown in Equation 2 (Rao *et al.*, 2013).

$$\Delta\Delta Ct = \Delta C(sample) - \Delta Ct(calibrator) \quad \text{.....Equation 2}$$

Later, the gene expression level can be estimated using Equation 3 (Rao *et al.*, 2013).

$$2^{-\Delta\Delta Ct} \quad \text{..... Equation 3}$$

Primers for the amplification of the *FLO5*, *EFG1*, *TGL4*, *YDC1*, *SPE2*, *1MA1* and cDNA for *S.boulardii* and *S.cerevisiae* were designed using Primer Express Software 444 (Applied Biosystems®) (Table 9). The *ACT1* gene for *S. boulardii* and *S. cerevisiae* was considered as a housekeeping gene to carry out in RT-PCR so as to have internal control. Target genes in *S. boulardii* comparing to *S. cerevisiae* genes were measured based on Comparative C_T method.

Table 9. Primers used in screening and quantitative Real-Time PCR

Gene Name	Primer Sequence (3'-5')
ACT1 *FW*	3'-GGTGTTACTCACGTCGTTCC-5'
ACT1 *RV*	3'-GAAGTCCAAGGCGACGTAAC-5'
FLO5 *FW*	3'-TGGACCGGTACTTTCACCTC-5'
FLO5 *RV*	3'-CACGGTTTCGTCAGTTGGTT-5'
EFG1 *FW*	3'-TCCCAGATAATGGATGCAGGA-5'
EFG1 *RV*	3'-AGCGTTGGCTTTAATCTTATTCT-5'
TGL4 *FW*	3'-ACTCCAACCAAGGGTGACAA-5'
TGL4 *RV*	3'-GCGGACGTAATGGAATACCG-5'

YDC1 *FW*	3'-GTTCTTTCTGGCTGGCTGAC-5'
YDC1 *RV*	3'-TGGCAGGGCCAAATATGTTC-5'
SPE2 *FW*	3'-CAAGCCGCTATCCATCAAA-5'
SPE2 *RV*	3'-TTCGTCGTCATCCTCGATGT-5'
IMA1 *FW*	3'-TGGACCACGTATTCACGAGT-5'
IMA1 *RV*	3'-TAGTTTCGTCGGAGGCATGT-5'

2.2.4. Aggregation Assessment

Sc and *Sb* were cultivated in YPD medium as described in section 2.2.1. 7 µl of cell suspension were observed under a bright-field Zeiss Axioplan microscope (Carl Zeiss MicroImaging). 30 images were captured using a CCD camera (Cool SNAPFX, Roper Scientific Photometrics). The number of aggregates and the number of cells per aggregate was calculated for each image using the Metamorph software.

2.2.5. Adhesion to human epithelium cells

The VK2/E6E7 human vaginal epithelial cell line (ATCC CRL-2616) were cultivated in 24-well polystyrene plates (Greiner), in keratinocyte-serum-free medium, containing 0.1ng/ml human recombinant epidermal growth factor (EFG), 0.05 mg/ml bovine pituitary extract and 44.1mg/l calcium chloride, until a density of $2.5x10^5$ cells/ml was reached after 24h of incubation. The culture medium was then removed and substituted by fresh culture medium. *Sb* and *Sc* cells, cultivated in YPD medium as described in section 2.2.1, were then added to each well, with a density of $12.5x10^8$ CFU/well. Then, cells were incubated at 37°C, 5% CO_2, for 30 min. Afterwards, each well was washed 3 times with 500 µL of PBS pH 7.4, following the addition of 500 µL of Triton X-100 0.5% (v/v) and incubation at room temperature for 15 min. The cell suspension in each well was then recovered and spread onto YPD agar plates by using spheres, and incubated at 30ºC for 48h, to determine CFU (Colony Forming Units) count, which represents the proportion of cells adherent to the human epithelium.

2.2.6. Biofilm Quantification

In order to assess the capacity of biofilm formation of *S. cerevisiae* and *S. boulardii* cells, the Presto Blue assay was used. Cells were grown in Sabouraud's dextrose broth ((SDB) containing 40 g glucose (Merck) and 10 g peptone (LioChem) per liter, pH 5.6) and collected at mid-

exponential phase. A cell suspension was prepared with an OD at 600 nm of 0.1. Cells were then inoculated in 96-well polystyrene titter plates (Greiner), which were previously filled with the appropriated medium, YPD, SDB at pH 5.6 or Roswell Park Memorial Institute (RPMI) 1640 growth medium (containing per 100 mL: 2.08 g RPMI 1640 (Sigma); 6.91 g 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma); 3.6 g glucose (Merck)), at pH 4 and 7, so as to have an initial OD600nm = 0.05±0.005. The design of the biofilm formation experiment shown in Figure 18.

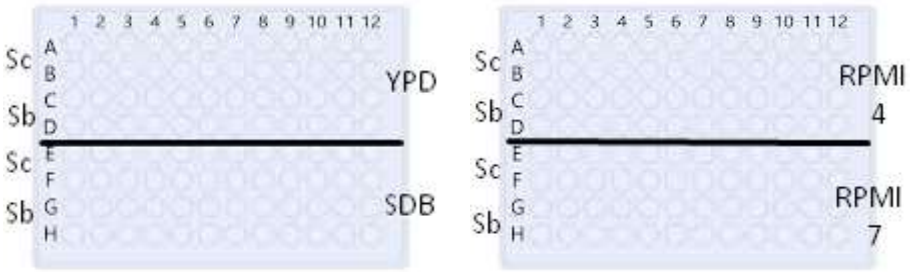


Figure 18. Schematic representation of the experimental design of 96-well polystyrene titter plates (Greiner) for biofilm determination.

Afterwards, cell suspensions were sealed with a membrane (Greiner Bio-One) and cultivated at mild orbital shaking (100 rpm), for 24h, at 30ºC. Subsequently, each well was washed two times with 100 µL of sterile PBS pH 7.4 [PBS contained per liter: 8 g NaCl (Panreac), 0.2 g KCl (Panreac), 1.81 g $NaH_2PO_4.H_2O$ (Merck), and 0.24 g KH_2PO_4 (Panreac)] to remove the cells that were not attached to the formed biofilm. Presto Blue reagent was prepared in a 1:10 solution in the medium used for biofilm formation, adding 100 µL of the solution to each well in the dark. Plates were incubated at 37ºC for 30 min. At the end of these processes, absorbance reading was determined in a microplate reader (SPECTROstar Nano, BMG Labtech) at the wavelength of 570 nm and 600 nm for reference.

2.3. Statistical Analysis

Statistical analysis of all data was performed using Microsoft EXCEL 2016. P-values were calculated performing one-way ANOVA tests on Microsoft® EXCEL 2016. P-values equal or inferior to 0,05 were considered statistically significant.

3. RESULT AND DISCUSSION

3.1. Contribution to the development of the ProBioYeastract Database

As the first stage of this study, ProBioYeastract Database was built, using the recently disclosed genome sequences of *S. boulardii* Biocodex and Unique 28 strains.

The contribution of this thesis to the database was the definition of the steps underlying the "Cross-strain Comparision" query.

As the beginning of the study, the Table of Cross-strain comparison of S. *boulardii* Biocodex, Unique 28 and S288C was built manually (Table 8), using individual queries available at the YEASTRACT and ProBioYeastract databases, as described in the Methodology chapter. The establishment of the sequencial steps required to reach this final table was done as an iterative process. The "Cross-strain Comparison" query allows the user to search for the Transcription Factors (TFs), predicted to be involved in the regulation of *S. boulardii* Biocodex and Unique 28 genes, but not in the homologous genes in *Sc* S288C, based on the occurrence of *Sc* TFs whose consensus binding site matches a subsequence of the promoter region of the genes. In the ProBioYeastract database, the input required is the names of ORF, so as to reach the cross-species comparison of *S. boulardii* Biocodex, Unique 28 and S288C.

3.2. New clues on the probiotic activity of *S. boulardii*, when compared to *S.cerevisiae*

3.2.1. Cross-strain promotor comparison of putative probiotic gene regulation

The obtained results from Cross-strain promotor comparison as shown in Table 8, aiming to find probiotic-related genes, collected from literature (Table 3), whose regulation in *Sb* is different from that in *Sc*. While performing this analysis, it was observed that some genes have two copies in *Sb*, but only one in *Sc*, namely *FLO5*, *CAR1*, and *PRO1* (in blue color in Table 8). Table 8 highlights the genes whose promoters share TF binding sites in *Sb* Biocodex and Unique 28 strains that do not exist in Sc S288C. Those 26 genes (out of the 83 analysed) are, thus, predicted to be differentially regulated in the *Sb* vs *Sc* strains. If this is the case, their differential expression may contribute to the observed probiotic activity of *Sb* strains, which is not present in *S. cerevisiae*.

Table 8. Design of Cross-strain promoter comparison table to help building of ProBioYeastract Database

Predicted Gene Names	Unique to *Sc*	Unique to *Sb* Biocodex	Unique to *Sb* Unique28	Unique to both *Sc* and active *Sc*	Unique both Biocodex and Unique28
Genes Related to Anti-toxin probiotic effect of *S. boulardii*, preventing or treating diarrhea and colitis caused by *C. difficile* infection					
PCR1	x	x	Gcn4p,lys14p, Rgt1p,Rgt3p, Rtg1p,Rtg3p Skn7p,Yap1p, Yap3p Abf1p,Bas1p,Hsf1p,Mcm1p, Ste12pAce2p,Swi5p, Ash1p, Rgt1p, Mcm1p	x	x
GLN3	x	x	Azf1p,Ime1p, Mcm1p Msn2p,Msn4pNrg1p,Rph1p Pho4p,Rlm1pYrr1p, Tda9p Ace2p, Swi5p, Hap2p Hap3p Hap4p, Hap5p	x	x
GAT1	x	Adr1p,Hsf1p, Sfl1p, Ste12p	Pho4p	x	Rlm1p

RRT12	x	x	The gene has no in Unique28	x	There is no comparison due to absence of gene in Unique 28
YSP3	x	x	x	x	x
Predicted genes related to anti-toxin effect of *S. boulardii* , preventing or treating diarrhea caused by *E.coli*					
PHO8	x	x	Aft2p, Aft1p, Bas1p, Gcn4p, Hsf1p, Ime1p, Mcm1p, Pdr8p,Rtg3p,Yap3p Ace2p, Swi5p	x	x
PRP3	x	x	Crz1p, Cup2p, Hap2p, Hap3p, Hap4p, Hap5p, Gis1p, Ime1p Msn2p	x	x
JIP4	x	x	Aft2p, Aft1p, Hsf1p, Mcm1p, Sfl1p, Ste12p, Yrr1p Ace2p, Swi5p, Cup2p	x	x
SNF1	Azf1p, Mcm1p Ecm22p, Upc2p, Uga3p, Com2p	Aft2p, Aft1p, Bas1p, Gcn4p, Cup2p, Gln3p, Gcn4p, Skn7p Ndt80p, Sum1p, Stp1p, Stp2p	Cat8p, Sip4p, Cbf1p, Met31p, Met32p,Swi4p, Ace2p, Swi5p	Upc2p	Gat1p, Gln3p, Gzf3p

SNM1	Adr1p, Rim101p	Ace2p, Swi5p, Azf1p, Crz1p, Gat1p, Gln3p, Gzf3p, Mcm1p, Stp2p, Xbp1p, Yrr1p, Haa1p Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Nrg1p, Rph1p	x	x	x
PEX29	Hcm1p	Crz1p, Ndt80p, Sum1p	Ace2p, Swi5p, Adr1p, Lys14p, Mbp1p, Mcm1p, Rlm1p, Yap1p	x	Cup2p, Hac1p, Tec1p, Xbp1p
CWC2	x	x	Bas1p, Gcn4p, Cbf1p, Crz1p, Fkh1p, Fkh2p, Gcn4p, Hac1p, Hsf1p, Pdr8p, Pho4p, Skn7p, Yap1p, Yap3p, Yrr1p, Tda9p	x	Gat1p, Gln3p, Gzf3p
VPS52	x	Bas1p, Gcn4p, Cup2p, Gat1p, Gln3p, Gzf3p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Ste12p, Stp1p, Stp2p, Xbp1p, Yap1p, Com2p	x	x	x

VPS72	x	x	Adr1p, Bas1p, Gcn4p, Cbf1p, Gat1p, Gln3p, Gzf3p, Hsf1p, Pdr8p, Pho4p, Sfl1p, Yap1p, Hot1p	x	x
RIB3	x	x	Azf1p, Gat1p, Gln3p, Gzf3p,Stp1p, Rim101p	x	x
PAC11	x	x	Ace2p, Swi5p, Adr1p, Mac1p, Yrr1p, Haa1p	x	x
Predicted genes related to Anti-toxin effect of *S. boulardii,* preventing or treating AAD related to pathogen infection					
FLO5	Aft2p, Aft1p, Arg80p, Gcn4p, Mac1p, Rlm1p, Sfl1p	Cst6p, Ime1p, Sko1p, Upc2p, Cad1p, Yap3p, Cin5p, Yap5p, Yrr1p, Rim101p	Haa1p, Gsm1p, Skn7p, Pho4p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Crz1p	Aft1p	x
FLO5	Aft2p, Aft1p, Arg80p, Gat1p, Gln3p, Gzf3p, Hsf1p,Mac1p, Rlm1p, Sfl1p	Crz1p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Met31p, Met32p, Skn7p, Gsm1p, Haa1p	Yap3p, Rpn4p, Cbf1p, Bas1p	Aft1p	Adr1p Pho4p
FLO8	x	Bas1p, Gcn4p, Crz1p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C,	x	x	x

		Hsf1p, Mac1p, Msn2p, Msn4p, Nrg1p, Rph1p, Rlm1p, Rpn4p, Skn7p, Tda9p, Com2p			
FLO9	x	Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Gln3p, Hac1p, Pho4p, Skn7p, Gsm1p, Ace2p, Swi5p	x	x	x
FLO10	x	x	x	x	x
FLO11	Gsm1p	x	Ace2p, Swi5p, Bas1p, Gcn4p, Cup2p, Hac1p	x	x
FIG2	x	Azf1p, Crz1p, Hap2p, Hap3p, Hap4p, Hap5p, Mcm1p, Ndt80p, Sum1p, Tda9p	x	x	x
EFG1	Ace2p, Swi5p, Azf1p, Gat1p, Gln3p, Gzf3p, Hac1p, Ste12p, Haa1p Com2p	x	x	Ace2p	Ecm22p, Upc2p, Hsf1p Lys14p, Tec1p

SAG1	x	x	Cst6p, Met31p, Met32p, Rlm1p, Swi4p, Rim101p Aca1p, Cst6p, Hac1p, Sko1p, Hap2p, Hap3p, Hap4p, Hap5p, Skn7p	x	x
Predicted genes related to antimicrobial effect of *S. boulardii,* preventing and treating IBD					
ACC1	x	x	Cbf1p,Hac1p,Mcm1p, Met4p,Pho4p,Sfl1p,Skn7p, Xbp1p, Yap1p,Haa1p	x	x
HFA1	RLm1p	Msn2p, Msn4p, Nrg1p, Rph1p, Gsm1p, Hap2p, Hap3p, Hap4p, Hap5p, Gis1p, Rph1p, YER130C	Adr1p, Bas1p, Gcn4p	Ace2p	Gat1p, Gln3p, Gzf3p
Predicted genes related to the trophic effect of *S. boulardii,* preventing of Lactose Intolerance					
MIG1	Azf1p, Cat8p, Sip4p, Hac1p, Gat1p, Gln3p, Gzf3p, Skn7p, Gsm1p	x	x	x	Bas1p, Crz1p, Ime1p, Mbp1p, Nrg1p, Rtg1p, Rtg3p, Stp2p, Tec1p

PGM1	Azf1p, Cst6p, Gat1p, Gln3p, Gzf3p, Hsf1p, Ste12p		x	Ste12p	Cup2p, Met4p, Nrg1p, Upc2p, Tda9p
CYC8	Rgt1p	Adr1p, Crz1p, Hsf1p, Mcm1p, Nrg1p, Rme1p Tec1p	x	x	x
GAL7	x	Cbf1p, Gcn4p, Pho4p, Sfl1p, Skn7p Ste12p Xbp1p,	x	x	x
PGM2	Pho4p	Ace2p, Swi5p, Cup2p, Gat1p, Gln3p, Gzf3p, Hap2p, Hap3p, Hap4p, Hap5p, Hsf1p, Ime1p, Ndt80p, Sum1p, Nrg1p, Skn7p, Gsm1p Ino2p, Ino4p	x	x	x
TUP1	Rtg1p, Rtg3p	x	Xbp1p, Mbp1p, Hac1p, Ace2p, Swi5p, Yap1p, Mcm1p, Gln3p	x	x

GAL1	x	x	Adr1p, Gcn4p, Mac1p, Ndt80p, Sum1p, Skn7p, Xbp1p, Yrr1p, Gsm1p, Usv1p Bas1p, Gcn4p, Hap2p, Hap3p, Hap4p, Hap5p	x	x
GAL10	x	Cup2p, Hac1p, Mcm1p, Ste12p, Ace2p, Swi5p	x	x	x
GAL2	Azf1p, Cbf1p, Gat1p, Gln3p, Gzf3p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Gln3p, Hsf1p, Met4p, Msn2p, Msn4p, Nrg1p, Rph1p, Com2p, Usv1p Rgt1p, Tec1p, Haa1p Cup2p	x	x	Met4p, Cbf1p Cup2p	Ace2p, Swi5p, Bas1p, Gcn4p, Hac1p, Hap2p, Hap3p, Hap4p, Hap5p, Mcm1p, Skn7p, Yap1p, Yap3p Sko1p, Ste12p, Stp2p
GAL4	x	x	x	x	x
GAL80	x	x	Adr1p, Hsf1p, Pho4p, Rpn4p, Skn7p, Bas1p, Gcn4p,	x	x

GAL3	x	Adr1p, Hac1p, Hap2p, Hap3p, Hap4p, Hap5p, Mac1p, Mcm1p	Gcn4p,Ste12p	x	x
Predicted genes promoters related to the trophic effect of *S.boulardii*, preventing and treating Obesity and Type II diabetes					
TGL2	x	x	Adr1p, Cat8p, Sip4p, Nrg1p, Rlm1p	x	x
TGL3	x	Adr1p, Ndt80p, Sum1p, Sfl1p, Ste12p, Tec1p, Uga3p, Bas1p, Gcn4p,	Hac1p, Ime1p, Msn2p, Msn4p, Nrg1p, Rph1p, Pho4p	x	x
TGL4	Adr1p, Azf1p, Cup2p, Ecm22p, Upc2p, Gcn4p, Lys14p, Rgt1p, Rlm1p, Upc2p, Gsm1p, Ste12p	x	x	Ste12p	Ace2p, Swi5p, Gln3p, Sfl1p
TGL5	Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Msn2p, Msn4p, Nrg1p, Rph1p, Pho4p, Mac1p Mbp1p, Rgt1p	x	x	Msn2p, Ste12p Swi4p	Adr1p, Azf1p, Ecm22p, Upc2p, Gcn4p, Gln3p, Hsf1p, Met31p, Met32p, Ndt80p, Sum1p,

	Rlm1p, Ste12p, Stp2p, Swi4p, Gsm1p, Com2p				Nrg1p, Stp1p, Stp2p, Tec1p, Upc2p, Yrr1p, Rim101p
AYR1	x	Cbf1p, Hac1p, Ste12p, Rim101p, Haa1p, Pho4p	x	x	x
TGL1	Tec1p	x	x	x	Ime1p, Mcm1p Nrg1p,
YJU3	x	x	x	x	x
YPC1	x	Cbf1p	Adr1p,Azf1p, Crz1p, Gat1p, Gln3p, Gzf3p, Mcm1p, Nrg1p, Rlm1p, Stp2p, Swi4p, Gsm1p Aft2p, Aft1p, Hsf1p	x	x
YDC1	Crz1p, Stp2p, Xbp1p, Haa1p Bas1p	x	x	x	Cbf1p
Predicted genes related to prevention of tight junction distribution of *S. boulardii,* treating diseases of Irritable bowel syndrome (IBS), gluten intolerance, gastroenteritis, and *H. pylori* infections					
STE11	Cup2p, Hac1p Mcm1p, Met31p, Met32p, Pho4p, Rgt1p	x	x	Met31p	Adr1p, Hap2p, Hap3p, Hap4p, Hap5p,

	Rlm1p				Hsf1p, Lys14p, Upc2p, Xbp1p, Com2p Bas1p, Gcn4p
STE7	x	Ace2p, Swi5p, Aft2p, Aft1p, Cbf1p, Crz1p, Hap2p, Hap3p, Hap4p, Hap5p, Met4p, Stp2p, Yrr1p	x	x	x
FUS3	x	x	Aft2p, Aft1p, Crz1p, Gat1p, Gln3p, Gzf3p, Fkh1p, Fkh2p, Hac1p, Swi4p, Yrr1p Xbp1p	Msn2p	Gcn4p
KSS1	Adr1p, Gcn4p, Gcr1p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Ndt80p, Sum1p, Rlm1p			Msn2p, Msn4p, Rph1p	Yrr1p
SSK2	x	x	Adr1p, Hsf1p, Mac1p, Mbp1p, Rlm1p, Skn7p	x	x
PBS2	Haa1p	x	Adr1p, Aft2p, Aft1p, Crz1p, Gat1p, Gln3p, Gzf3p, Gcr1p,	x	x

			Hac1p, Mac1p, Stp1p, Stp2p, Tec1p, Upc2p Ste12p		
HOG1	Ecm22p, Upc2p, Gln3p, Ndt80p, Sum1p, Rlm1p, Upc2p, Zap1p Skn7p, Adr1p	x	x	Skn7p Rlm1p	Crz1p Lys14p Mac1p, Mcm1p Met31p, Met32p Pdr1p, Pdr3p, Pho4p, Ste12p, Rim101p Hot1p
BCK1	Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Rlm1p, Skn7p, Ste12p, Yrr1p, Tda9p	Crz1p,Cup2p, Hac1p, Hsf1p, Ime1p, Mcm1p, Mot3p, Stp1p, Stp2p, Xbp1p	The gene has no in Unique28	Msn2p	There is no comparison due to absence of gene in Unique 28
SMK1	Azf1p, Gat1p, Gln3p, Gzf3p, Mac1p, Skn7p, Stp1p, Stp2p, Swi4p, Ume6p Upc2p, Com2p, Ndt80p, Sum1p, Aft2p, Aft1p,	x	x	Fkh2p Ndt80p, Sum1p Ume6p	Gln3p Rim101p, Pho4p, Bas1p, Crz1p

	Ace2p, Swi5p				
Predicted genes promoters related to increasing immune defense in the gut by *S. boulardii*, aiming to treat or prevent allergic diseases					
SPE2	Abf1p Adr1p, Azf1p, Gln3p, Ime1p, Pdr1p, Pdr3p, Pdr8p, Yrr1p	x	x	x	Cbf1p, Crz1p,Cup2p, Gis1p, Msn2p, Msn4p, Rph1p, YER130CHac1p,Pho4p Ste12p, Yap1p Bas1p, Cst6p
SPE3	x	x	x	x	x
CAR1	Bas1p, Gcn4p, Ecm22p, Upc2p, Mac1p, Rlm1p, Ste12p, Ume6p, Upc2p, Xbp1p, Yrr1p, Gsm1p,Yap3 Gis1p, Rph1p, YER130C, Mcm1p	x	x	Gcn4p Ume6p	Ace2p, Swi5p, Azf1p Crz1p, Pho4p, Hac1p
CAR1	x	x	x	x	x
CAR2	Azf1p, Cup2p, Bas1p, Gcn4p,	x	x	Msn2p Mcm1p	Aft2p, Aft1p, Hsf1p,Gcr1p, Skn7p

	Mcm1pGis1p, Msn2p, Msn4p, Rph1p, YER130C, Rph1p				
PUT2	x	Adr1p,Arg80p, Bas1p, Gcn4p,Crz1p, Cst6p, Cup2p, Hac1p, Mcm1p, Sko1p, Uga3pHap2p, Hap3p, Hap4p, Hap5p, Yrr1p	x	x	x
PUT1	Adr1p, Azf1p, Cbf1p, Gat1p, Gzf3p, Gcn4p, Mac1p, Met4p, Pho4p Rgt1p, Stp2p, Yrr1p, Gsm1p, Haa1p	x	x	Cbf1p, Gat1, Gln3p Gcn4p, Met4p, Stp2p	Rim101p Ste12p,Tec1p Met31p, Met32p Hsf1p,Hac1p, Gcr1p,Yap1p, Cad1p, Yap3p, Cin5p, Yap5p
PRO1	x	x	Ecm22p, Upc2p, Ime1p,Yap1p Yrr1p	x	x

PRO1	Usv1p, Stp1p, Stp2p, Sfl1p, Mcm1p, Hsf1p, Gat1p, Gzf3p, Adr1p Ace2p, Swi5p	Crz1p, Mac1p, Mbp1p, Pdr1p, Pdr3p, Rox1p, Gsm1p	Com2p, Upc2p, Swi4p, Pho4p, Met4p, Ecm22p, Cbf1p, Aft2p, Aft1p	Ace2p	Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Nrg1p, Rph1p, Ume6p
PRO2	Hcm1p, Mbp1p, Mcm1p, Ndt80p, Sum1p, Swi4p, Upc2p, Rim101p	x	x	Gcn4p	Gat1p, Gln3p, Gzf3p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C Nrg1p, Rph1p, Hot1p,Ace2p, Swi5p, Ste12p
PRO3	x	x	Adr1p, Cat8p, Sip4p Cbf1p, Pho4p, Ste12p, Stp2p, Yrr1p, Rim101p Haa1p	x	x
Predicted gene promoters related to immunomodulation effect of *S. boulardii*, aiming to treat or prevent gluten intolerances and celiac diseases					
IMA1	Swi4p, Aft2p, Aft1p, Azf1p Cbf1p,Crz1p	x	x	Cin5p	Rim101p, Upc2p, Ste12p, Skn7p,Msn2p, Msn4p, Nrg1p, Rph1p,Hac1p, Gcr1p,Ecm22p, Adr1p

Cup2p, Dal81p, Dal82p, Pho4p,Cad1p, Yap3p, Cin5p, Yap5p	Yrr1p,Gcn4p

3.2.2. Differential gene expression of selected genes: *S. boulardii* vs *S. cerevisiae*

To evaluate if the observed differences in the *Sb* and *Sc* gene promoter regions result in differences at the level of gene expression, the transcript levels of 6 selected genes, representative of the various mechanisms of probiotic activity exhibited by *S. boulardii*, was measured through RT-PCR. Gene expression was assessed in exponentially growing cell, cultivated in YPD medium, and YPD with sodium cholate, which mimics, to some extent, human intestinal environmental conditions.

The expression of each selected *Sb* gene was analyzed by RT-PCR, and compared to the corresponding homolog in *S. cerevisiae* (used as a reference), in triplicate.

The expression of four genes, *FLO5*, *TGL4*, *YDC1* and *SPE2*, was found to be down-regulated in *Sb* cells, when compared to *Sc*, while two genes, *EFG1* and *IMA1*, were found to be up-regulated in *Sb* vs *Sc*, in cells cultivated in YPD medium (Figure 19). In YPD supplemented with cholate, the results were similar, with the exception of *IMA1*, whose up-regulation was not observed (Figure 20).

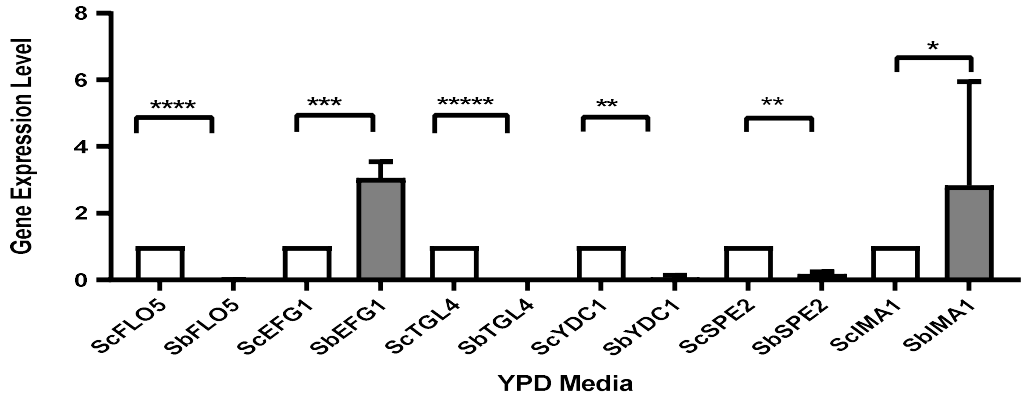


Figure 19.Distribution of gene expression level of selected genes by using YPD medium in *S.boulardii* and *S. cerevisiae* was taken into account as a reference value, identified by RT-PCR analysis to be related to the regulation of genes. The genes found as *EFG1* and *IMA1* (up-regulated) and *FLO5*, *TGL4*, *YDC1* and *SPE2* (down-regulated). Error bars represent the corresponding standard deviations. ***** P<0,00001; **** P<0,0001, ***P<0.001; ** P<0,01; * P<0,05

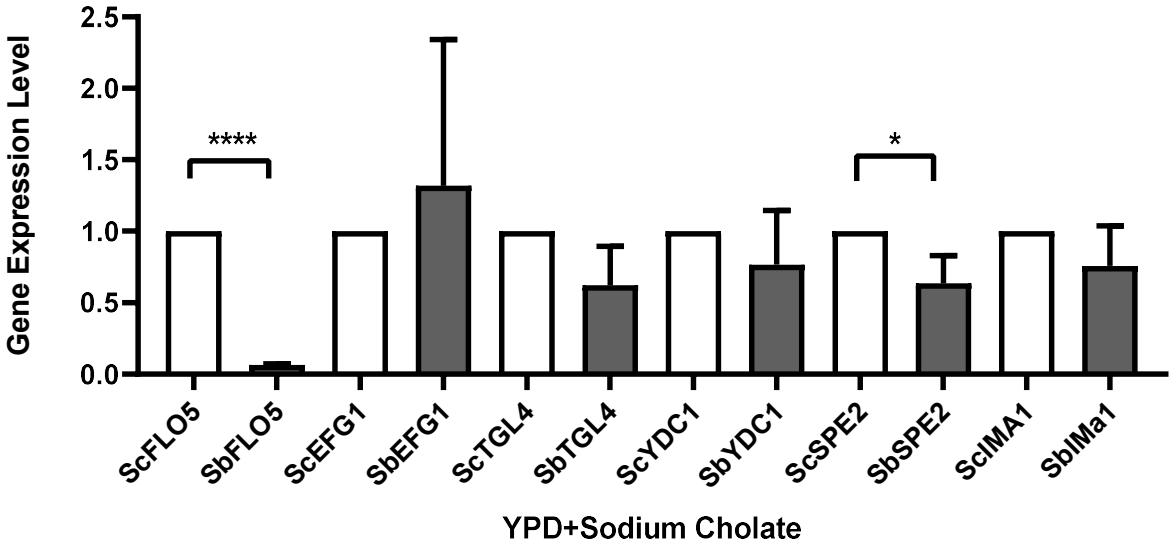


Figure 20.Distribution of gene expression level of selected genes by using YPD+Sodium cholate that mimics human gastrointestinal system, in *S.boulardii*, and *S. cerevisiae* was taken into account as a reference value, identified by RT-PCR analysis to be related to regulation of genes. The genes found as *EFG1* (up-regulated) and *FLO5*, *TGL4*, *YDC1* and *SPE2* (down-regulated). Error bars represent the corresponding standard deviations. **** P<0,0001;* P<0,05

Considering the down-regulated genes, it is reasonable to hypothesize that it is not their activity that makes *S. boulardii* a probiotic organism, when compared to *S. cerevisiae*. This appears to be the case for *FLO5*, that contributes to flocculation and adhesion in *S. cerevisiae* (Govender *et al.*, 2008), *TGL4*, that contributes to lipid degradation (Fietto *et al.,* 2004; Rajakumari and Daum 2010), *YDC1*, that encodes a dehydroceramide hydrolase, involved in sphingolipid degradation (Vandenbosch *et al.*, 2013) and *SPE2*, involved in the synthesis of polyamines (Balasundaram *et al.*, 1994).

Considering the up-regulated genes, *EFG1* and *IMA1*, their activity may indeed contribute to the probiotic phenotype of *Sb*. Interestingly, Vandenbosch et al. (2013) reported the decreased of biofilm formation upon the deletion of *EFG1* in S288C, suggesting that it plays a role in this process, which is known to be important for the probiotic activity of *Sb*. In *S. cerevisiae*, Efg1 is a protein required for maturation of 18S rRNA, so its link to biofilm formation is likely indirect, through the control of the expression of biofilm related proteins. This hypothesis, of course, requires further confirmation.

Figure 21.Distribution of the putative TF binding sites in the promoter regions of the *EFG1* genes in *S. boulardii* Biocodex (ORF *KO01_01677*) and Unique28 (ORF *AB282_01893*) strains, as obtained in the "Search TF" query of the ProBioYeastract database.

Interestingly, when we look at the promoters of the *EFG1* genes in *S. boulardii* Biocodex and Unique28 strains, they share the precise locus for the binding of the TFs that are displayed in Figure 21. These transcription factors binding sites exist only in the promoter of the *Sb EFG1* genes, but not in the promoter of the *Sc EFG1* gene, suggesting that at least one of them controls the differential expression of these genes in *Sb* strains, compared to *Sc*. Among these TFs there are two controlling sterol biosynthesis, Ecm22 and Upc2, one regulating lysin biosynthesis, Lys14, one controlling the heat shock response, Hsf1, and one involved in filamentation and biofilm formation, Tec1.

IMA1, on the other hand, encodes a major isomaltase in *Sc* and *Sb*, whose activity may be very important in the fight against gluten intolerance and celiac diseases. However, data presented for *IMA1* gene (with high standard error) in this study does not provide a statistically significant result, and thus, this experiment should be repeated (Figure 19).

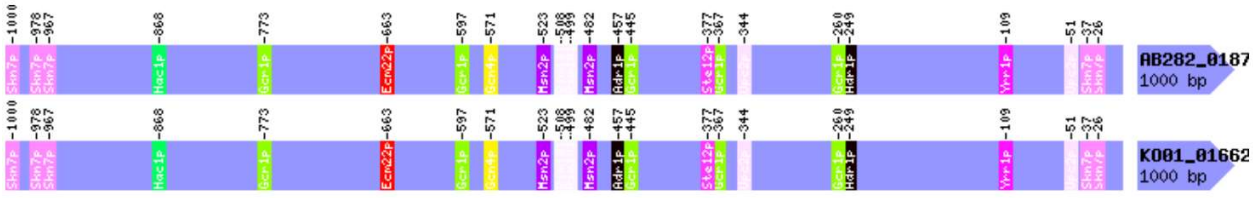


Figure 22.Distribution of the putative TF binding sites in the promoter regions of the *IMA1* genes in *S. boulardii* Biocodex (ORF *KO01_01662*) and Unique28 (ORF *AB282_01878*) strains, as obtained in the "Search TF" query of the ProBioYeastract database.

Interestingly, when we look at the promoters of the *IMA1* genes in *S. boulardii* Biocodex and Unique28 strains, they share the precise locus for the binding of the TFs that are displayed in Figure 22. These transcription factors binding sites exist only in the promoter of the *Sb IMA1* genes, but not in the promoter of the *Sc IMA1* gene, suggesting that at least one of them controls the differential expression of these genes in *Sb* strains, compared to *Sc*. Among these TFs there

are eight controlling stress response, Msn2, Msn4, Skn7, Rim101, Yrr1, Hac1, Gcn4 and Rph1, three related to the control of glucose repression/derepression, Nrg1, Adr1 and Gcr1, two controlling sterol biosynthesis, Ecm22 and Upc2, and one involved in filamentation and mating, Ste12. Since *IMA1* encodes an isomaltase the glucose related transcription factors may be particularly relevant.

In general, it is possible to conclude that the expression of selected genes is indeed different in *Sb*, when compared to *Sc*, confirming the promoter analysis outcome. It also shows that the expression of these genes is different depending on the growth media used, which suggest that further experiments should be conducted in media that more faithfully mimics the gastrointestinal tract (Fietto *et al.*, 2004).

3.3. *S.boulardii* exhibits higher aggregation, adhesion to human epithelial cells and biofilm formation than *S. cerevisiae*

Given the importance of adhesion in the probiotic activity of *S. boulardii*, and the indication that the expression of *EFG1*, related to biofilm formation, is higher in *Sb*, when compared to non-probiotic *Sc* strains, we decide to test if *Sb* Biocodex displays higher ability than *Sc* to aggregate, adhere to human epithelial cells and form biofilm.

The obtained results show that *S. boulardii* has the ability to aggregate more frequently than *S. cerevisiae* (Figure 23). Based on bright-field microscopy, it was possible to assess the percentage of cells that we found as aggregates, versus the total number of cells per image. *S. boulardii* was found to display higher levels of cell-to-cell aggregation (55.6 %), when compared to *S. cerevisiae* (36.5 %).

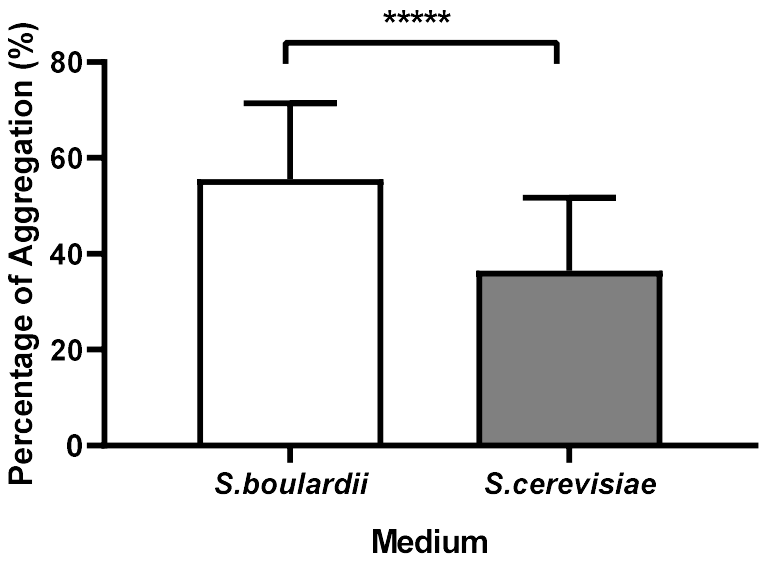


Figure 23.The percentage of aggregation in *S.boulardii* and *S.cerevesia* cells, *S.boulardii* is formed more aggregation when grown in YPD medium, compared to *S. cerevisiae* under the same conditions. Standard deviation being represented by the error bars. ***** P<0,00001.

The ability of *S. boulardii* cells to adhere to human epithelial cells was also analyzed, and compared to that of *S. cerevisiae* (Figure 24). It was found that indeed the percentage of adhering *S. boulardii* cells (74.3 %) is much bigger that that of *S. cerevisiae* cells (16 %).

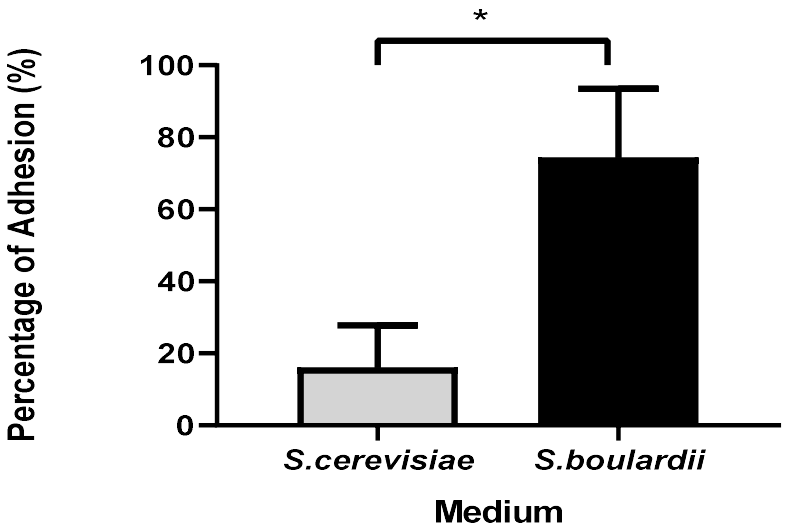


Figure 24. Percentage of Adhesion in *S.boulardii* and *S.cerevisiae* cells, *S.boulardii* is formed more adhesion when grown in YPD medium, compared to *S. cerevisiae* under the same conditions. Error bar represents the corresponding standard deviation, * P<0,05.

Finally, biofilm formation in polystyrene surfaces by *S. boulardii* and *S. cerevisiae* was evaluated, using the PrestoBlue cell viability assay in four growth media: YPD, SDB and RPMI pH 4 and RPMI pH 7.

Except for cells growing in YPD medium, in all cases *S. boulardii* cells were found to form larger biofilms than *S. cerevisiae* cells (Figure 25). Interestingly, the difference was found to be particularly strong in RPMI medium, which mimics the composition of human fluids.

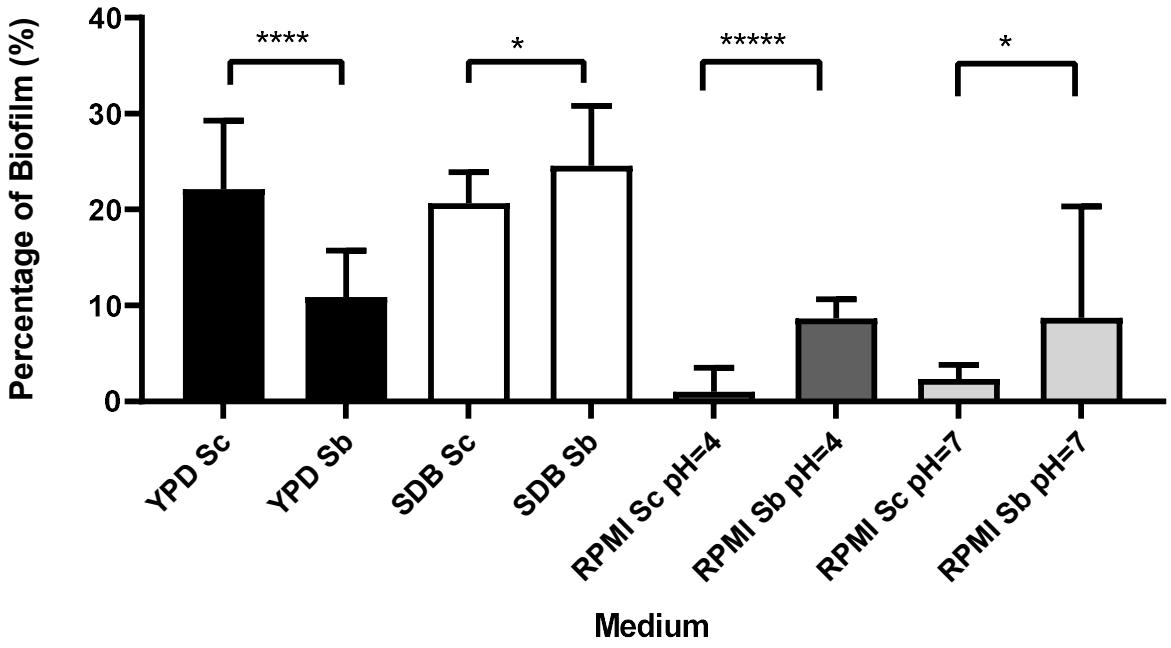


Figure 25.Biofilm formation followed by Presto Blue Cell Viability Assay and measurements of absorbance at 570 nm and 600 nm (reference) for the *S.cerevisiae* and *S. boulardii*, when compared to different medium (YPD, SDB, RPMI both pH=4 and pH=7). Error bars represent the corresponding standard deviations. ***** P<0,00001; **** P<0,0001; * P<0,05.

To the best of our knowledge, this is the first demonstration of the higher adhesion levels of *S. boulardii* cells, when compared to *S. cerevisiae*. The fact that *S. boulardii* displays higher adhesiveness, particularly to human epithelial cells, than *S. cerevisiae* may contribute to its longer period of persistence in the human gut. Besides, it may also contribute to the role of *S. boulardii* in preventing dysbiosis in the gut, providing a healthy balance (homeostasis) between intestinal epithelial cells. Indeed, biofilms of probiotics have been shown to be a protective barrier and provide colonization resistance against pathogenic bacteria (Kechagia *et al.*, 2013). Furthermore, Moré and Vandenplas (2018) reported that *S. boulardii* provides a physical barrier effect and colonization resistance. In support of these, one *in vivo* study on germ-free mice

conducted by Tiago and collegues (2012) has shown that four different strain of *S. boulardii* as a probiotic have ability to exert its antimicrobial effect by adhering to intestinal mucus membrane and removing pathogens by flow inhibiting their adhesion to the intestine.

Altogether, these results provide interesting clues on the molecular basis of the probiotic activity of *S. boulardii*, which is not displayed by *S. cerevisiae.*

4. Conclusion & Future Perspectives

S. boulardii is a well-known probiotic yeast that can be used in the treatment or prevention of specific gastrointestinal tract diseases, such as IBS, AAD and gluten intolerance. On the other hand, non-*boulardii S. cerevisiae* strains, although sharing 99% homology at the level of the genome sequence, do not display probiotic activity (Douradinha *et al.*, 2014). The molecular basis of this different behavior remains to be established.

In this context, this study aimed to evaluate if the registered differences between probiotic and non-probiotic *S. cerevisiae* strains relies on differences at the level of gene transcription regulation. As a result of the *in silico* cross-strain promotor analysis, comparing *S. boulardii* Biocodex and Unique28 strains with *S. cerevisiae* S288C strain, the expression of 26 probiotic-related genes was manually predicted to be controlled by different transcription factors in probiotic vs non-probiotic strains. Additionally, this work motivated the construction of the ProBioYeastract and the pipeline from this thesis was used as a basis for a new functionality in the database. Moreover, this work featured the initial development of contents of ProBioYeastract database that is still under construction. So far, the molecular mechanism of *Saccharomyces* probiotic strains are still unclear. The completion of the ProBioYeastract database may shed light on the better genetic and mechanistic understanding of the gene expression regulation of probiotics which could lead to exert their probiotic features. In the future, ProBioYeastract database might provide an useful mechanism for grouping a list of probiotic genes depending on their transcription factor binding sites, and compare it with non-probiotics *Sc* strains.

The up-regulation of *EFG1* and *IMA1* genes in *S. boulardii* Biocodex, when compared to *S. cerevisiae* BY4741, was observed, leading us to propose that their overexpression in *S. boulardii* strains may underly its probiotic activity. Given the importance of *EFG1* in biofilm formation, the ability of *S. boulardii* Biocodex, when compared to *S. cerevisiae* BY4741, to aggregate, adhere to human epithelial cells and form biofilms was evaluated and shown to be higher in all cases. Further studies are, however, needed to elucidate more details in this area and to verify the hypothesis proposed in this study.

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