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

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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*

INTRODUCTION

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).

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).

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 30º C, *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.

The hypothesis that this study 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 study contributed to its development.

We carried out 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.

METHODOLOGY

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”.

Gene Expression Analysis

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

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.

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.

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.

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 and the retrotranscription program used. In the second step, Real-Time PCR reactions were prepared for each sample 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.

Aggregation Assessment

Sc and *Sb* were cultivated in YPD medium. Afterwards,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.

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 2.5x105 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 previous section, were then added to each well, with a density of 12.5x108 CFU/well. Then, cells were incubated at 37°C, 5% CO2, 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.

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

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 $NaH_2PO4.H2O$ (Merck), and 0.24 g KH2PO4 (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.

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.

RESULTS & DISCUSSIONS

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 work 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.

New clues on the probiotic activity of S. boulardii, when compared to S.cerevisiae: Cross-strain promotor comparison of putative probiotic gene regulation

The obtained results from Cross-strain promotor comparison, aiming to find probiotic-related genes, collected from literature 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* 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*.

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 1). In YPD supplemented with cholate, the results were similar, with the exception of *IMA1*, whose up-regulation was not observed (Figure 2).

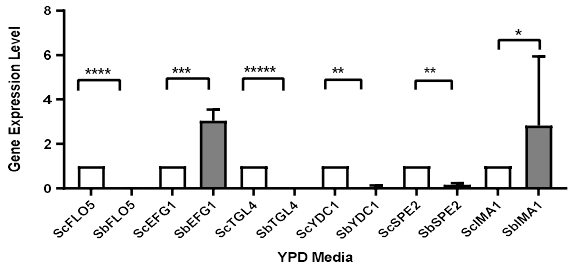

Figure 1 | Distribution of gene expression level of selected genes by using YPD medium in *S.boulardii* and *S. cerevisiae* was taken into account as a reference value, identified by RT-PCR analysis to be related to the regulation of genes. The genes found as *EFG1* and *IMA1* (up-regulated) and *FLO5*, *TGL4, YDC1* and *SPE2* (down-regulated). Error bars represent

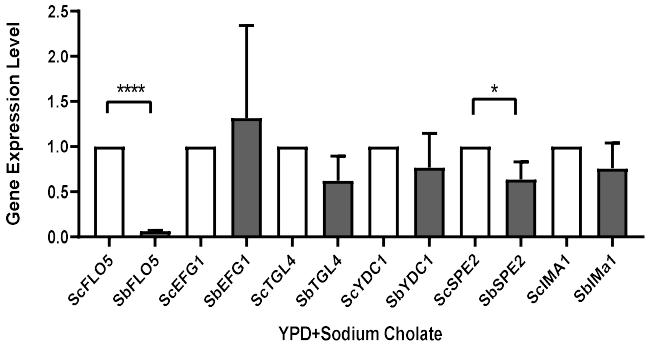

Figure 2 | Distribution of gene expression level of selected genes by using YPD+Sodium cholate that mimics human gastrointestinal system, in S.boulardii, and S. cerevisiae was taken into account as a reference value, identified by RT-PCR analysis to be related to regulation of genes. The genes found 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.

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 3-A. 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 1).

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 3-B. 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).

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 4).

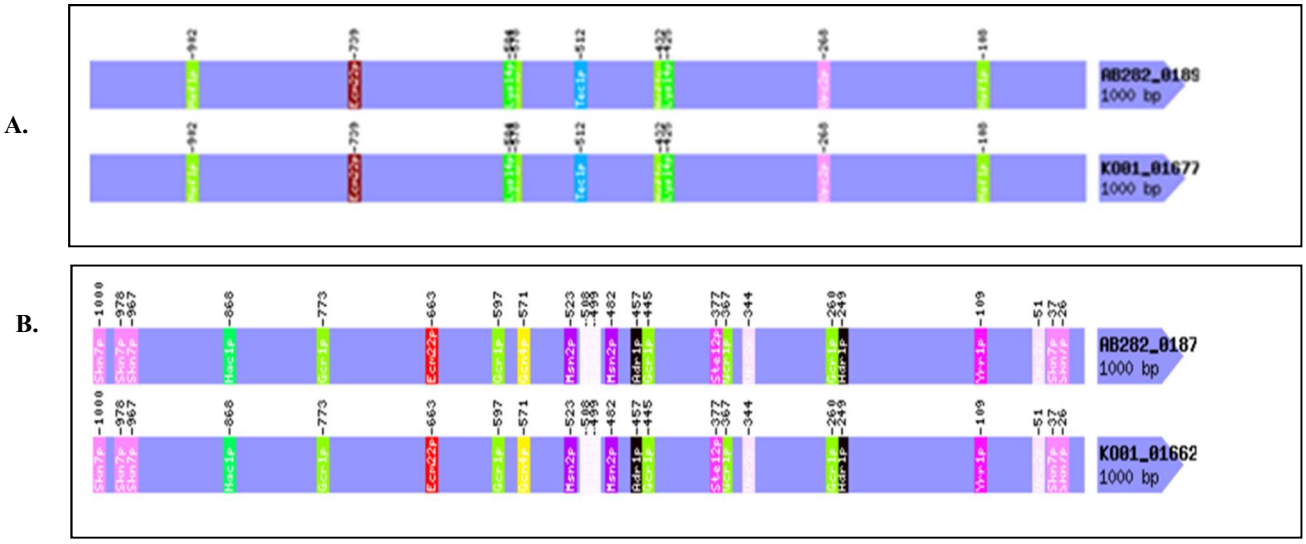

Figure 3| (A) Distribution of the putative TF binding sites in the promoter regions of the EFG1 genes in *S. boulardii* Biocodex (ORF KO01_01677) and Unique28 (ORF AB282_01893) strains, as obtained in the "Search TF" query of the ProBioYeastract database. (B) Distribution of the putative TF binding sites 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.

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 %).

The ability of *S. boulardii* cells to adhere to human epithelial cells was also analyzed, and compared to that of *S. cerevisiae* (Figure 5). It was found that indeed the percentage of adhering *S. boulardii* cells (74.3 %) is much bigger that that of *S. cerevisiae* cells (16 %).

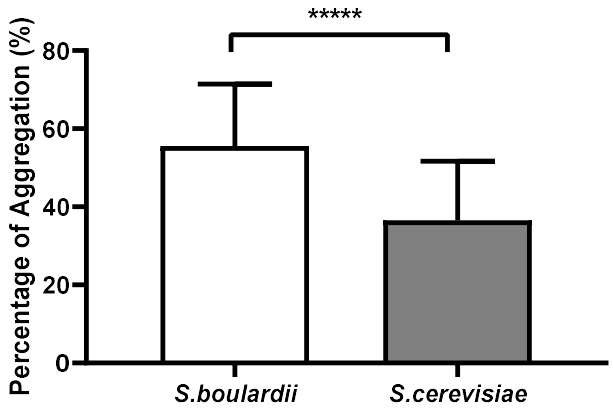

Figure 4 | The percentage of aggregation in *S.boulardii* and *S.cerevesia* cells, *S.boulardii* is formed more aggregation when grown in YPD medium, compared to *S. cerevisiae* under the same conditions. Standard deviation being represented by the error bars. ***** P<0,00001.

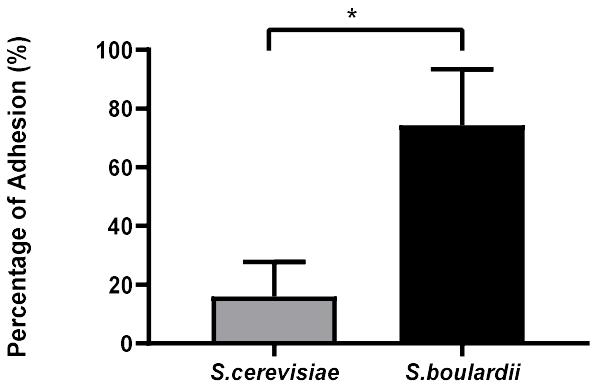

Figure 5 | Percentage of Adhesion in *S.boulardii* and *S.cerevisiae* cells, *S.boulardii* is formed more adhesion when grown in YPD medium, compared to *S. cerevisiae* under the same conditions. Error bar represents the corresponding standard deviation, * P<0,05.

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 6). Interestingly, the difference was found to be particularly strong in RPMI medium, which mimics the composition of human fluids.

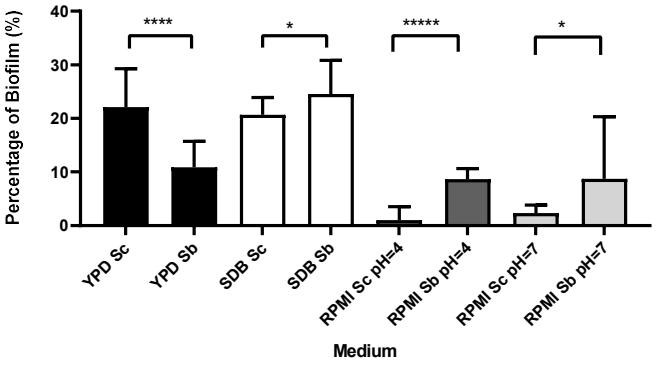

Figure 6 | Biofilm formation followed by Presto Blue Cell Viability Assay and measurements of absorbance at 570 nm and 600 nm (reference) for the *S.cerevisiae* and *S. boulardii*, when compared to different medium (YPD, SDB, RPMI both pH=4 and pH=7). Error bars represent the corresponding standard deviations. ***** 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.*

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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