Antioxidant properties and global metabolite screening of the probiotic yeast *Saccharomyces cerevisiae var. boulardii*

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Abstract

BACKGROUND: *Saccharomyces cerevisiae var. boulardii* is the only yeast species with probiotic properties. It is considered to have therapeutic significance in gastro-intestinal disorders. In this paper, a comparative physiological study between this yeast and *Saccharomyces cerevisiae* (BY4742) was performed by evaluating two prominent traits of probiotic species, responses to different stress conditions and antioxidant capacity. A global

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metabolite profile was also developed in order to identify which therapeutically important secondary metabolites are produced.

RESULTS: *S. cerevisiae var. boulardii* showed no significant difference in growth patterns but greater stress tolerance when compared with *S. cerevisiae*. It also demonstrated a 6-10-fold greater antioxidant potential (judged by the DPPH assay), with 70-fold higher total phenolic content and 20-fold higher total flavonoid content in the extracellular fraction. These features were clearly differentiated by principal component analysis and further elucidated by metabolite profiling. The extracellular fraction of the *S. cerevisiae* var. *boulardii* cultures was found to be rich in polyphenolic metabolites viz. vanillic acid, cinnamic acid, phenyl ethyl alcohol (rose oil), erythromycin, amphetamine and vitamin B6 which results in the antioxidant capacity of this strain.

CONCLUSION: This study presents a new perspective of differentiating the two genetically related strains of yeast, *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae var. boulardii* by assessing their metabolome fingerprints. In addition to the correlation of the phenotypic properties with the secretory metabolites of these two yeasts, the work also emphasizes the potential to exploit *S. cerevisiae* var. *boulardii* in the industrial production of these metabolites.

Keywords: *Saccharomyces cerevisiae* var. *boulardii* NCYC 3264, probiotic, *Saccharomyces cerevisiae* BY4742, stress-tolerance, antioxidant capacity, global metabolite profiling

Introduction

Saccharomyces cervesiae var. boulardii, has been used as an adjunctive therapy for treatment of infectious gastroenteritis or in the prevention and cure of antibiotic associated diarrhea (AAD). It was isolated from the skin of lychee and mangosteen in Indochina and has gained popularity as an alternative remedy in Europe, Africa, and South America [1, 2] for AAD, acute diarrhea in children, traveler's diarrhea and irritable bowel syndrome [3, 4].

Activities of *S. cerevisiae var. boulardii* include modification of host-signalling pathways involved in inflammatory and non-inflammatory intestinal diseases [5], inhibition of bacterial toxins and trophic effects on the intestinal mucosa [3]. In these studies, researchers have carried out experiments on crude extracts of *S. cerevisiae var. boulardii* to assess its anti-inflammatory, antitoxin [6] and anti-proliferative [7] properties.

However, the taxonomic characterization of *S. cerevisiae var. boulardii* has been controversial. Some groups have reported this yeast to be a separate species from *S. cerevisiae* on account of its therapeutic value, lack of ability to produce ascospores and its apparent inability to utilize galactose [8, 9]. Interestingly, a later study by McCullough *et al* reported that *S. cerevisiae var. boulardii* is capable of utilizing galactose [10]. Based on nuclear DNA (nDNA)-nDNA reassociation data, Cardinali and Martini located this yeast outside the sensu stricto *S. cerevisiae* cluster [11]. On the contrary, Molnar *et al* found that *S. cerevisiae var. boulardii* and the type strains of *S. cerevisiae* shared the same profile in a Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) analysis [12]. However, another study was able to distinguish between the two strains using this method [13]. Although, McCullough *et al* concluded that Restriction Fragment Length Polymorphism of the PCR-amplified intergenic transcribed spacer regions (ITS1-5.8S rDNA-ITS2) was not capable of differentiating *S. cerevisiae var. boulardii* from *S. cerevisiae* [1]. In another study, microsatellite length polymorphism of clinical

isolates, laboratory strains and industrial strains of *S. cerevisiae* resulted in a distinct pattern of *S. cerevisiae var. boulardii*, which could be distinguished from *S. cerevisiae* [14]. Comparative genome hybridization (CGH) and other physiological analyses revealed that *S. cerevisiae var. boulardii* strains differ significantly from laboratory strains of *S. cerevisiae* at both the genomic and physiological levels with regard to sporulation, individual chromosome and gene copy numbers, ability for pseudohyphal switching and survival at low acid pH. The latter two features have a direct bearing on the probiotic nature of *S. cerevisiae var. boulardii* [15].

Apart from the molecular typing techniques, the local stress responses of probiotics entering the gastrointestinal tract against factors like variations in temperature and pH, presence of gut enzymes, bile salts and organic acids are able to distinguish the phenotypic attributes of this yeast. These responses are complex processes involving the production of number of metabolites, intermediates, proteins and enzymes at a different level or with different activity compared to those observed before stress exposure. Evidence of heat shock proteins, osmotic shock related proteins, glutathione and thioredoxin playing vital role in protection and repair of damaged cell components and induced levels of enzymes as a response of re-organised metabolic flux in response to stress exposure has been well-documented in yeast [16].

While over half of the last century was spent in commercial application of S. *cerevisiae var. boulardii* whole yeast formulations in treatment of diarrhea, and scientific interests on its modus operandi for therapeutic use [17], not much research has been channeled into exploring the presence of biomolecules such as metabolites or polypeptides and possibility of their potential industrial applications. In this study, we aimed to compare two related yeast (*S. cerevisiae var. boulardii* and *S. cerevisiae*) by characterising both the strains with particular reference to the probiotic and antioxidant properties of the former. *S. cerevisiae* was chosen for this comparison

because it is extremely well characterised biochemically and genetically. For example, the genome of this organism is essentially fully determined [18]. This study has also identified which biomolecules are present in the extracellular and intracellular extracts of these two yeasts.

Materials and methods

Strains, media and Chemicals

YPD broth and separate media ingredients (e.g. yeast extract, dextrose, peptone and agar) were purchased from Difco™ BD Biosciences, USA. All other chemicals and solvents were purchased from Sigma-Aldrich Co., India. *S. cerevisiae* var. *boulardii* NCYC 3264 was procured from National Collection of Yeast Cultures (NCYC, Norwich, United Kingdom). The *S. cerevisiae* S288c-derived laboratory strain BY4742 (MATα his3Δ, leu2Δ, lys2Δ, ura3Δ) was a gift from Dr. Himanshu Sinha's laboratory (Department of Biological Sciences, Tata Institute of Fundamental Research, India).*Culture growth conditions*

Yeast starter cultures (5 ml) were grown overnight in a rotary shaker (200 rpm) at 30 °C in YEPD medium containing 1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, and 2% (*w*/*v*) glucose. Overnight cultures were diluted to OD_{600} = 0.1 and inoculated in YEPD broth filled wells in microtitre plates. Plates were incubated at 30 °C with shaking. For anaerobic growth, wells were overlaid with paraffin oil and incubated without shaking at 30 °C [19]. Growth curves were generated by sampling and reading the OD_{600} value after every 15mins until the cultures reached stationary phase using Freedom Evo 75 liquid handling and robotics station (Tecan®, Switzerland) equipped with a microplate reader. The maximum specific growth rate (k) was

calculated by plotting the OD_{600} values *vs* time fitted to the logistic growth equation in GraphPad Prism 6.0 (GraphPad Software Inc, CA, USA) as follows:

$OD=OD_{max}OD_0/((OD_{max}-OD_0)\ exp^{(-kt)}+OD_0)$

Where k is the maximum specific growth rate constant, t is the time taken in the exponential phase of the growth curve, OD_0 and OD_{max} are the optical densities at t = 0 and t = t_{max} (stationary phase) respectively. The optimum doubling time (t_d) was calculated as $t_d = \log_e 2/k$.

In vitro stress tolerance tests mimicking gastro-intestinal environment

Yeast cultures (OD_{600} = 1.0) were heat shocked by shifting incubation temperatures from 30°C to 37°C (normal body temperature), 39°C (fever condition) and 45°C (an extreme heat stress) in YEPD medium for 1 h. Aliquots were collected after 0, 10, 20, 30, 40, 50 and 60 min of incubation and the cell viability was determined microscopically by using a Neubauer chamber and vital staining with methylene blue [20]. For experiments on simulated gastro-intestinal stress, overnight cultures (OD_{600} = 1.0) were harvested by centrifugation at 3000*g* for 5 min, washed with distilled water once, and incubated at 37 °C for 1 h in (*i*) a simulated gastric environment constituted by an aqueous solution containing 3 g/L pepsin (3200–4500 U/mg) and 5 g/L NaCl, pH 2.0 [21], (*ii*) a simulated intestinal environment aqueous solution containing 1 g/L pancreatin (903 U/mg) and 5 g/L NaCl, pH 8.0, and (*iii*) exponentially grown yeast cells were diluted to an OD_{600} of 1.0 and 5 µL of this dilution was used to spot-inoculate solid YPD medium supplemented with 0.25%, 0.5%, 0.75% and 1% w/v concentrations of bile salts. Plates were visualized after 48 h at 30 °C and 37 °C.

Preparation of cell free extract (CFE) and ethyl-acetate fraction (EAF)

Sterile YPD broth (pH 6.5±0.2 at 25°C) was inoculated with 1 % (V/V) of the overnight grown culture of the two strains of yeast, *S. cerevisiae var boulardii* NCYC 3264 and *S. cerevisiae* BY4742 and incubated at 30 °C for 16-18 h approximately until the same stage of growth (mid-log phase). The overnight cultures were subjected to centrifugation at 5000g for 5 min at 4 °C. The supernatant was used as the CFE for assessing the total phenolic content (TPC), total flavonoid content (TFC) and the antioxidant capacity assays. A fraction of the supernatant was also extracted using ethyl acetate at 1:3 ratio (v/v) for 3 h agitation followed by filtration and concentration at 50°C maintained in water bath for 60 min *in vacuo* using a rotavac. The dried solute was resuspended in 40% ethanol and recovered from rotavac.

Evaluation of the antioxidant potential

Total phenolic content (TPC): The total polyphenolic content was determined colorimetrically using the Folin-Ciocalteau (FC) method according to Singleton *et al*. [22] with some modifications. The test sample (0.5 ml) was mixed with 0.2 ml of FC reagent and allowed to stand for 10 min, then 0.6 ml of 20% (*w/v*) sodium carbonate was added and mixed. The reaction mixture was incubated at 40 °C for 30 min. Absorbance of the reaction mixture was measured at 765 nm using UV/VIS spectrophotometer (Shimadzu Inc., Japan). Gallic acid was used as standard to construct the calibration curve (Pearson's correlation coefficient: $R^2 = 0.994$) and the total level of phenolics for each sample was expressed as milligram gallic acid equivalents per gram of extract ($mgGAE.g^{-1}$).

Total flavonoid content (TFC): Total flavonoid content was determined by using the aluminium chloride colorimetric method as described by Willet [23] with some modifications. Aqueous ethanol extracts (0.5 mL), 10% (*w/v*) aluminium chloride (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (4.3 mL) were mixed. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm using a using UV/VIS spectrophotometer (Shimadzu Inc., Japan). Quercetin was used as the standard and a calibration curve (Pearson's correlation coefficient:$R^2 = 0.986$) was constructed. Total flavonoid content was expressed as milligram quercetin equivalents per gram extract (mg QE. g^{-1}).

DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging ability: The hydrogen atom or electron-donation ability of the corresponding extracts was measured from the bleaching of a purple-coloured methanol solution of DPPH [24]. The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical, was determined by the method described by Dan *et al* [25] with some modifications. Briefly, an aliquot of 1 ml of 0.1 mM DPPH solution in ethanol and 0.5 ml of extract were mixed. The mixture was shaken vigorously followed by incubation at room temperature for 30 min in the dark. The absorbance (Abs) was measured at 517 nm. Butylated hydroxytoluene **(**BHT) was used as positive control and DPPH mixture without any sample served as blank. Percentage of inhibition (I %) of DPPH radical was determined using formula as below:

$$I\% = [Abs_{(blank)} - Abs_{(sample)}]/Abs_{(blank)} \times 100$$

Trolox equivalent antioxidant capacity assay (TEAC): Experiments were carried out according to Biskup *et al* [26]. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)

and potassium persulfate ($K_2S_2O_8$) were dissolved in distilled water to prepare the ABTS radical solution with a final concentration of 7 mM ABTS and 2.45 mM $K_2S_2O_8$ followed by incubation in the dark at room temperature for 12-16 h before use in order to produce ABTS radical ($ABTS^{\bullet+}$) . The solution was diluted with distilled water to an absorbance of 0.7 ± 0.2 at 734 nm prior to use. Trolox (6-hydroxy- 2,5,7,8-tetramethylchroman-2- carboxylic acid) was used as standard. Working standard concentrations from 0-20 mM and extracts were added to diluted $ABTS^{\bullet+}$ solution and absorbance was measured 6 min after mixing at 734nm. $ABTS^{\bullet+}$ radical inhibition was expressed as µM Trolox equivalent antioxidant capacity (TEAC).

Global metabolite profiling

This method was carried out according to the protocol by Villas-Bôas *et al* [27] with slight modifications.

Sample Preparation: Yeast cultures (5 ml) were grown under laboratory conditions (aerobic, 30 °C) until mid-log phase (OD_{600} = 0.5) were quenched with 20 ml of 60% (v/v) methanol – buffer (PIPES 3 mM, EDTA 3 mM, pH 7.0) solution at -40 °C, resulting in a final methanol concentration of 50% (v/v), followed by centrifugation at 770*g* for 20 min at -20 °C to separate the biomass. The biomass concentration was measured between 0.7 – 1.0 g cell dry weight per litre (CDW l^{-1}). Aliquots of the supernatant were evaporated to dryness under vacuum using a vacuum centrifuge (Eppendorf™, Germany) and stored as the extracellular fraction at -80 °C until the derivatisation step. Pellet was further treated for overall intracellular metabolite extraction i.e. amino acids, organic acids, nucleotides, peptides, sugars, sugar alcohols and sugar phosphates by chloroform : methanol : buffer (CMB) extraction [27] and fatty acids by pure methanol extraction [28]. CMB extraction was carried out by resuspending the pellet in 5 ml cold

chloroform (−40 °C) followed by addition of 2.5 ml cold methanol (−40 °C) and 2ml ice-cold buffer (PIPES, 3 mM; EDTA, 3 mM; pH 7.2) sequentially under manual shaking. The suspension was agitated vigorously at 300 r.p.m on a platform shaker for 45 min at 4 °C. Separation of the chloroform and the aqueous phase was induced by centrifugation (770*g*, 20 min, −20°C). The upper aqueous phase, containing the polar metabolites from the intracellular fraction, was collected without disturbing the pellet of the cell debris on top of the lower chloroform phase (lipophilic metabolites). A second extraction step was repeated and the extract was pooled with the upper aqueous phase in order to increase the concentration of the polar metabolites. Pellet was further extracted with 2.5ml pure methanol followed by freezing in liquid nitrogen for 10mins. The frozen suspension was thawed in an ice-bath and centrifuged at 770*g* for 20 min at −20 °C. Supernatant was collected and pooled with a second extract. Extracts (supernatants) of the intracellular fractions were evaporated to dryness using a vacuum centrifuge (Eppendorf™, Germany) and stored at -80 °C until the derivatisation step.

Derivatisation: Methyl chloroformate (MCF) - alkylation derivatisation was carried out according to Villas-Bôas *et al*. (2003b) [29] with some modifications. Briefly, vacuum dried samples were resuspended in NaOH (200 µl of a 1 M solution) and mixed with 34 µl of pyridine and 167 µl of methanol, followed by addition of 20 µl MCF and vortexed for 30 s carried out twice. Chloroform (400 µl) was added to the mixture to separate the MCF derivatives. After vigorous mixing, $NaHCO_3$ (400 µl of 50 mM) was added to partition the aqueous layer. Upper aqueous layer was discarded and the chloroform phase was subjected to GC-MS analysis.

GC-MS Analysis: Samples were analyzed on a Varian 450 gas chromatograph equipped with a Varian 220 mass spectrometer (ion trap), using a VF-5MS capillary column (30 m × 0.25 mm thick, 0.25 mm). Helium was used as mobile phase at 1.0 ml min^{-1}, with a splitless injection

volume of 1.0 µl at 200 °C, a transfer liner at 220 °C and an ion trap at 150 °C. The mass spectrometer was operated in SCAN mode from 45 to 650 *m/z*. The column temperature was initially held at 45 °C for 2 min. Thereafter, the temperature was raised with a gradient of 9 °C min^{-1} until it reached 180 °C. This temperature was held for 5 min. Next, the temperature was raised with a gradient of 40 °C min^{-1} until it reached 220 °C. The temperature was again held for 5 min. Finally, the temperature was raised with a gradient of 40 °C min^{-1} until it reached 240 °C, which was held for 11.5 min. Data handling was carried out using WORKSTATION, Version 6.9.1 software equipped with National Institute of Standards & Technology (NIST) mass spectrum (MS) database. The metabolites were identified using the MS database.

Statistical analysis

Univariate analyses were conducted using GRAPHPAD PRISM 6.0 (GraphPad Software Inc, CA, USA) for Windows. Analysis of Variance (ANOVA) and Pearson's correlation coefficients were performed to compare the data. All determinations were done at least in triplicate and were reported as means. The confidence limits used in this study were based on 95% ($p < 0.05$). Principal component analysis (PCA) was performed using SPSS Statistics 20 (IBM Corporation, USA) for data processing and STATISTICA 7.1 (Dell Inc., USA) for output.

Results and discussion

The two strains show similar growth patterns under laboratory and simulated in vivo conditions

The growth curves of the organisms tested under normal laboratory conditions (30 °C, aerobic) and at simulated *in-vivo* temperature and oxygen availability (37°C, anaerobic) are shown in Figure 1. Both the yeast strains showed no significant difference in growth rates under the two

conditions (Table 1) although the maximum optical density values (OD_{max}) were significantly different between the strains under both conditions (Table 1). The optimum doubling times (t_d) were measured at the period of maximum growth rate (k). The similar growth kinetics pattern of both the yeasts possibly explains why neither strain is able to colonise as a permanent part of the gut flora in healthy individuals and is eliminated from the system within 3-5 days after its oral administration is discontinued [30]. The growth assessment of the two strains aided in deciding the sampling time points for attaining certain biomass level before carrying out the metabolome experiments in the later part of this study.

S. cerevisiae var. boulardii is more stress tolerant than S. cerevisiae

S. cerevisiae var boulardii NCYC 3264 and *S. cerevisiae* BY4742 showed significant difference in percentage viability upon heat treatment for 1 h at 37 °C (p value = 0.0078) and 39 °C (p value = 0.0051). Greater resistance was observed when compared to that of *S. cerevisiae* at both temperatures (Figure 2a, b). However, both the strains showed comparable decrease overtime with a 57.6% final viability for *S. cerevisiae var. boulardii* NCYC 3264 and 50.3% for *S. cerevisiae* BY4742 at 45 °C (p value = 0.0141) (Figure 2c). Viability levels under simulated gastric environment (pH 2.0) were indistinguishable for the first 10 min, but after 15 min, *S. cerevisiae var boulardii* NCYC 3264 appeared to be more resistant, maintaining its cell viability at about 57.6% with *S. cerevisiae* BY4742 falling to about 32% after 60 min (p value = 0.0037) (Figure 2d). Exposure to the simulated intestinal environment (pH 8.0) showed a slight difference in percentage viability (p value = 0.0419) for both the strains with a final value of 39% and 32.3% for *S. cerevisiae var boulardii* NCYC 3264 and *S. cerevisiae* BY4742 respectively (Figure 2e). *S. cerevisiae var boulardii* NCYC 3264 also exhibited a higher

tolerance than *S. cerevisiae* BY4742 for the bile tolerance assay (Figure 3). Studies with *Bifidobacterium spp.* probiotic strains show resistance to cholate concentration from 0.125 to 1.2% [31]. Other published literature suggest 0.3% as the minimum concentration to define an organism as resistant to bile salts [32] and the majority of yeast species grow in the presence of 0.75% bile salts. This study partly conforms with these observations with a sensitivity of *S. cerevisiae var boulardii* NCYC 3264 at 1% cholate concentration at both 30°C and 37°C but *S. cerevisiae* BY4742 being sensitive to 0.5% cholate at both the temperatures (Figure 3). Interestingly, a counter observation was made by Fietto *et al* with no impact on cell viability of both *S. cerevisiae var boulardii* and *S. cerevisiae* by the simulated intestinal environment and both the strains showing sensitivity to bile salt concentrations more than 0.1-0.15% [33]. This study did not specify the growth stage studied (and it is not clear which growth stage, if any, most closely recapitulates the prevalent phase in the mammalian gut) whereas our study used mid-log phase cells. Potential differences such as this in the experimental procedures might account for the apparent discrepancy in the results.

S. cerevisiae var boulardii demonstrates antioxidant potential

The TPC of cell free extracts (CFE) and ethyl acetate fractions (EAF) of *S. cerevisiae var boulardii* NCYC 3264 were found to be significantly higher (~ 70 fold) than those of *S. cerevisiae* BY4742 (Table 2). A similar pattern was observed for the DPPH radical scavenging activity with the percentage inhibition of the DPPH radical in the order of BHT (control) > CFE of *S. cerevisiae var boulardii* > EAF of *S. cerevisiae var boulardii* > CFE of *S. cerevisiae* > EAF of *S. cerevisiae* (Table 2). The total flavonoid content for both the extracts of *S. cerevisiae var boulardii* were ~20-fold higher than that of *S. cerevisiae* BY4742 (Table 2). The TEAC values

were also higher in case of *S. cerevisiae var boulardii* with no detectable activity for both the extracts of *S. cerevisiae* (Table 2). The data suggests that *S. cerevisiae var boulardii* has broad antioxidative properties. In this study, ethyl acetate extraction of cultures was carried out as a means to generate polyphenol-rich fractions having bioactivities which could be attributed to their antioxidant properties [34, 35]. A study by Pu *et al* also reported EAF of plant extracts as the fraction to demonstrate highest antioxidant activity [36]. However, there was no significant difference between the activities shown by the CFE and EAF of *S. cerevisiae var boulardii* in this study.

Multivariate analysis distinguished between S. cerevisiae var. boulardii and S. cerevisiae

Principal component analysis was employed as a means of multivariate analysis in a reduced dimensional scaling approach to identify global similarities and differences between the two strains of *S. cerevisiae var. boulardii* NCYC 3264 and *S. cerevisiae* BY4742 under two different conditions (aerobic, 30 °C and anaerobic, 37 °C). The inter-relationship among the growth kinetic, probiotic and antioxidant attributes i.e. doubling time (t_d), maximum specific growth rate constant (k), viability response under heat (37 °C, 39 °C and 45 °C), simulated gastric (pH 2.0), intestinal (pH 8.0) and bile salt (0.5% w/v) stress and antioxidant capacity (TPC, TFC and DPPH radical scavenging activity), were also considered as variables in the analysis. A two-component construct of all the variables demonstrating a cumulative 92.97% of the total variance based on the eigenvalues of correlation matrix of all the components (Supplementary Table S1; Supplementary Figure S1) is illustrated in Figure 4 and Table 3. The first (PC1) and second (PC2) principal components represent 73.62% and 19.35% of the total variance respectively. Figure 4a and 4b depict the projection of the attributes (variables) and strains under different

conditions (cases) respectively. A clear separation of the probiotic and antioxidant attributes represented as a cluster along the X-axis from the growth kinetic parameters along the Y-axis was achieved (Figure 4a and Table 3a). The proximity of *S. cerevisiae var. boulardii* to the probiotic and antioxidant cluster (Figure 4b and Table 3b) clearly differentiates the strain from *S. cerevisiae* which is shown to be away from the cluster and hence negatively correlated with these two particular attributes. Thus, the two strains were well differentiated in the PCA supporting the general conclusion that they have different antioxidant profiles.

Global metabolite profiling revealed antioxidant molecules

A total of 22 metabolites in the extracellular and 18 metabolites in the intracellular fractions were identified in *S.cerevisiae var boulardii* NCYC 3264 (Table 4a) and seven metabolites in the extracellular and 35 metabolites in the intracellular fractions were identified in *S. cerevisiae* BY4742 (Table 4b) using a semi-qualitative analysis based on their fragmentation pattern in mass-spectroscopy (MS) and chromatographic retention time (R_t). The extracellular fraction of *S.cerevisiae var boulardii* NCYC 3264 demonstrated the presence of aromatic metabolites like phenyl ethyl alcohol (orange oil), mephaneine, cymene, hydroxycinnamic acid, cinnamic acid, naphthalenol (golden yellow), quinoline, erythromycin, vanillic acid and vitamin B6 in addition to the tricarboxylic acid cycle (TCA) metabolites such as succinate, acetate, citrate [37-39] and other central carbon metabolites such as amino acids and sugars (Table 4a). The intracellular fraction, on the other hand, contained largely intermediary central carbon metabolites (Table 4a). The extracellular fraction of *S. cerevisiae* BY4742 showed mostly TCA cycle organic acids such as citric acid, succinic acid, malic acid and fumaric acid along with lactic acid, glycerol and D-glucose, which was present as the substrate in growth media (Table 4b). The intracellular metabolites identified were proteinogenic and non-proteinogenic amino acids, organic acids,

sugars, and phosphorylated sugars representing key parts of central metabolism (Table 4b). However, most of the intracellular metabolites identified for both the yeasts were in the low abundance region of the chromatogram suggesting a possible loss due to leakage of metabolites during sampling or insufficient cell biomass at the start of experiment [40]. This study showed clear differences in the extracellular and intracellular metabolomes between the two yeasts with an abundance of aromatic metabolites in the extracellular fraction of *S.cerevisiae var. boulardii* NCYC 3264. The smaller number of central carbon metabolites in the intracellular fraction of *S.cerevisiae var. boulardii* NCYC 3264 when compared with *S.cerevisiae* BY4742 might be explained by the masking of weak intensity signals generated by the low concentrations of these metabolites in presence of higher concentrations of organic, inorganic acid and aromatic metabolites in the system. There was some overlap in the metabolites across the two metabolomes and between both the strains (Table 4). Some of this commonality between the extracellular and intracellular metabolite fractions may be partly due to the metabolic overflow [41, 42]. The pool of aromatic metabolites in the extracellular fraction of *S.cerevisiae var. boulardii* NCYC 3264 reflects the possibility of different role of these metabolites in this environment than inside the cell. A similar finding was reported by Granucci *et al* in *S. cerevisiae* CEN.PK113-7D strain where they speculated the extracellular medium as a storage place for some metabolites with roles other than central metabolism of the cell e.g. cell-to-cell communication, metabolic regulation and detoxification [42]. The presence of these compounds accounts for the antioxidative and probiotic property exhibited by *S.cerevisiae var. boulardii* NCYC 3264. However, it remains unclear what the benefits of producing such a large range of antioxidant molecules to this strain are. Nevertheless, the data indicate the possibility of exploring the potential of producing these metabolites on a larger, industrial scale from this strain.

Conclusions

This study addressed the characterization of *S. cerevisiae* var. *boulardii* NCYC 3264 in comparison with *S. cerevisiae* with respect to probiotic and antioxidant properties. The two strains are markedly different in these properties, despite close genetic similarities. Based on this dataset, a global screening of the metabolite profiles was performed for the two strains which revealed a variety of polyphenolic metabolites e.g. vanillic acid, cinnamic acid, phenyl ethyl alcohol, erythromycin and pyridoxine (vitamin B6). Many of these compounds have antioxidant properties and are likely to contribute to the previously reported probiotic nature of *S. cerevisiae* var *boulardii*. In addition, some have potential commercial significance as fragrances, aroma and flavour compounds in food and cosmetic industries in addition to their therapeutic value. For example, vanillic acid has been associated with a number of pharmacological activities (such as inhibiting snake venom [43, 44], hepatoprotective activity [45] and apoptosis [46, 47]) but its main application is for its pleasant creamy odour and taste leading to its widespread use in fragrances and licensing as a food additive (FAO/WHO Expert Committee on Food Additives, JECFA no. 959). Cinnamic acid is used mainly in manufacturing methyl, ethyl, and benzyl esters for the perfume and flavour industry and is a precursor molecule for manufacturing sweetener aspartame via enzyme-catalysed amination to phenylalanine [48]. Phenyl ethyl alcohol or rose oil (PEA) is an aromatic alcohol used as an ingredient in favour and perfumery particularly where the aroma of rose is desired [49]. There have been reports on production of PEA from a thermotolerant strain of *S. cerevisiae* (Ye9-612), highlighting the commercial potential of this metabolite [50]. Erythromycin is a potent 14-membered macrolide antibiotic active against pathogenic Gram-positive bacteria [51, 52]. Pyridoxine (vitamin B6) is manufactured as oral supplements in the pharmaceutical sector which is necessary for the activation of glycine in the initial stages of heme production *in vivo* [53] and is prescribed to treat a number of illnesses and deficiency

symptoms such as sickle-cell anemia and nervous disorders like Carpal-Tunnel syndrome [54-56]. The detection of these bioactive compounds in the metabolite fingerprints of the extracellular metabolome of *S. cerevisiae* var. *boulardii* suggests the possibility of using the strain for industrial scale production of these metabolites. However, before the viability of this can be fully assessed an in-depth biochemical study to elucidate the pathways (including key enzymes and intermediates) is required. Taken together, this study paves the path to understanding why *S. cerevisiae* var. *boulardii* is physiologically and metabolically different from baker's yeast in spite of its similar genetic make-up and provides a useful "baseline" for the assessment of any potentially probiotic yeasts discovered in the future. The data may also be useful in comparative studies between the various commercial preparations of *S. cerevisiae* var. *boulardii* which are sold worldwide allowing an assessment of the effects of formulation, storage etc on their probiotic properties.

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References

1. McCullough MJ, Clemons KV, McCusker JH and Stevens DA, Species Identification and Virulence Attributes of Saccharomyces boulardii (nom. inval.). *Journal of clinical microbiology* **36**:2613-2617 (1998).

2. Penna FJ, Filho LA, Calcado AC, Junior HR and Nicolli JR, [Up-to-date clinical and experimental basis for the use of probiotics]. *Jornal de pediatria* **76 Suppl 1**:S209-217 (2000).

3. Im E and Pothoulakis C, The intestinal microbiota: Equilibrium and disordersRecent advances in Saccharomyces boulardii research. *Gastroentérologie Clinique et Biologique* **34**:S62-S70 (2010).

4. Gareau MG, Sherman PM and Walker WA, Probiotics and the gut microbiota in intestinal health and disease. *Nature reviews Gastroenterology & hepatology* **7**:503-514 (2010).

5. Czerucka D and Rampal P, Experimental effects of Saccharomyces boulardii on diarrheal pathogens. *Microbes and infection / Institut Pasteur* **4**:733-739 (2002).

6. Pothoulakis C, Review article: Anti-inflammatory mechanisms of action of Saccharomyces boulardii. *Alimentary pharmacology & therapeutics* **30**:826-833 (2009).

7. Chen X, Fruehauf J, Goldsmith JD, Xu H, Katchar KK, Koon H-W, Zhao D, Kokkotou EG, Pothoulakis C and Kelly CP, Saccharomyces boulardii Inhibits EGF Receptor Signaling and Intestinal Tumor Growth in Apc(min) Mice. *Gastroenterology* **137**:914-923 (2009).

8. McFarland LV, Saccharomyces boulardii is not Saccharomyces cerevisiae. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **22**:200-201 (1996).

9. Mitterdorfer G, Kneifel W and Viernstein H, Utilization of prebiotic carbohydrates by yeasts of therapeutic relevance. *Letters in applied microbiology* **33**:251-255 (2001).

10. McCullough MJ, Clemons KV, McCusker JH and Stevens DA, Species identification and virulence attributes of Saccharomyces boulardii (nom. inval.). *Journal of clinical microbiology* **36**:2613-2617 (1998).

11. Cardinali G and Martini A, Electrophoretic Karyotypes of Authentic Strains of the Sensu Stricto Group of the Genus Saccharomyces†. *International Journal of Systematic and Evolutionary Microbiology* **44**:791-797 (1994).

12. Molnar O, Messner R, Prillinger H, Stahl U and Slavikova E, Genotypic Identification of Saccharomyces Species using Random Amplified Polymorphic DNA Analysis. *Systematic and Applied Microbiology* **18**:136-145 (1995).

13. Mitterdorfer G, Mayer HK, Kneifel W and Viernstein H, Clustering of Saccharomyces boulardii strains within the species S. cerevisiae using molecular typing techniques. *Journal of Applied Microbiology* **93**:521-530 (2002).

14. Hennequin C, Thierry A, Richard GF, Lecointre G, Nguyen HV, Gaillardin C and Dujon B, Microsatellite typing as a new tool for identification of Saccharomyces cerevisiae strains. *Journal of clinical microbiology* **39**:551-559 (2001).

15. Edwards-Ingram L, Gitsham P, Burton N, Warhurst G, Clarke I, Hoyle D, Oliver SG and Stateva L, Genotypic and physiological characterization of Saccharomyces boulardii, the probiotic strain of Saccharomyces cerevisiae. *Applied and environmental microbiology* **73**:2458-2467 (2007).

16. Estruch F, Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiology Reviews* **24**:469-486 (2000).

17. Łukaszewicz M, *Saccharomyces cerevisiae var. boulardii – Probiotic Yeast*. Prof. Everlon Rigobelo (Ed.) InTech (2012).

18. Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H and Oliver SG, Life with 6000 genes. *Science (New York, NY)* **274**:546, 563-547 (1996).

19. Pawar V, Jingjing L, Patel N, Kaur N, Doetsch PW, Shadel GS, Zhang H and Siede W, Checkpoint kinase phosphorylation in response to endogenous oxidative DNA damage in repair-deficient stationary-phase Saccharomyces cerevisiae. *Mechanisms of ageing and development* **130**:501-508 (2009).

20. Mills DR, DIFFERENTIAL STAINING OF LIVING AND DEAD YEAST CELLS. *Journal of Food Science* **6**:361-371 (1941).

21. Charteris WP, Kelly PM, Morelli L and Collins JK, Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. *Journal of applied microbiology* **84**:759-768 (1998).

22. Singleton VL, Orthofer R and Lamuela-Raventós RM, [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, in *Methods in enzymology*. Academic Press, pp 152-178 (1999).

23. Willett WC, Balancing life-style and genomics research for disease prevention. *Science (New York, NY)* **296**:695-698 (2002).

24. Gulluce M, Sahin F, Sokmen M, Ozer H, Daferera D, Sokmen A, Polissiou M, Adiguzel A and Ozkan H, Antimicrobial and antioxidant properties of the essential oils and methanol extract from Mentha longifolia L. ssp. longifolia. *Food Chemistry* **103**:1449-1456 (2007).

25. Dan Y, Lee WC, Mahmud R, Pillai S, Perumal S and Ismail S, 3rd International Conference on Biotechnology and Food Science (ICBFS 2012), April 7-8, 2012Antioxidant Activities of Essential Oil of Psidium Guajava L. Leaves. *APCBEE Procedia* **2**:86-91 (2012).

26. Biskup I, Golonka I, Gamian A and Sroka Z, Antioxidant activity of selected phenols estimated by ABTS and FRAP methods. *Postepy higieny i medycyny doswiadczalnej (Online)* **67**:958-963 (2013).

27. Villas-Boas SG, Hojer-Pedersen J, Akesson M, Smedsgaard J and Nielsen J, Global metabolite analysis of yeast: evaluation of sample preparation methods. *Yeast (Chichester, England)* **22**:1155-1169 (2005).

28. Maharjan RP and Ferenci T, Global metabolite analysis: the influence of extraction methodology on metabolome profiles of Escherichia coli. *Analytical biochemistry* **313**:145-154 (2003).

29. Villas-Boas SG, Delicado DG, Akesson M and Nielsen J, Simultaneous analysis of amino and nonamino organic acids as methyl chloroformate derivatives using gas chromatography-mass spectrometry. *Analytical biochemistry* **322**:134-138 (2003).

30. Kelesidis T and Pothoulakis C, Efficacy and safety of the probiotic Saccharomyces boulardii for the prevention and therapy of gastrointestinal disorders. *Therapeutic advances in gastroenterology* **5**:111-125 (2012).

31. Ruiz L, Margolles A and Sánchez B, Bile resistance mechanisms in Lactobacillus and Bifidobacterium. *Frontiers in Microbiology* **4**:396 (2013).

32. Gilliland SE, Staley TE and Bush LJ, Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. *Journal of dairy science* **67**:3045-3051 (1984).

33. Fietto JL, Araujo RS, Valadao FN, Fietto LG, Brandao RL, Neves MJ, Gomes FC, Nicoli JR and Castro IM, Molecular and physiological comparisons between Saccharomyces cerevisiae and Saccharomyces boulardii. *Canadian journal of microbiology* **50**:615-621 (2004).

34. Garcia-Salas P, Morales-Soto A, Segura-Carretero A and Fernandez-Gutierrez A, Phenolic-compound-extraction systems for fruit and vegetable samples. *Molecules (Basel, Switzerland)* **15**:8813-8826 (2010).

35. Oboh G RJ, *Antioxidant in Foods: A New Challenge for Food Processors.* Nova Science Publishers Inc., New York, US (2007).

36. Pu W, Wang D and Zhou D, Structural Characterization and Evaluation of the Antioxidant Activity of Phenolic Compounds from Astragalus taipaishanensis and Their Structure-Activity Relationship. *Scientific Reports* **5**:13914 (2015).

37. Sigler K, Knotkova A, Paca J and Wurst M, Extrusion of metabolites from baker's yeast during glucose-induced acidification. *Folia microbiologica* **25**:311-317 (1980).

38. Stoppani AO, De Favelukes SL and Conches L, Formation of succinic acid in baker's yeast through the citric acid cycle. *Archives of biochemistry and biophysics* **75**:453-464 (1958).

39. Wurst M, Sigler K and Knotkova A, Gas chromatographic determination of extracellular metabolites produced by baker's yeast during glucose-induced acidification. *Folia microbiologica* **25**:306-310 (1980).

40. Smart KF, Aggio RB, Van Houtte JR and Villas-Boas SG, Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography-mass spectrometry. *Nature protocols* **5**:1709-1729 (2010).

41. Giardina BJ, Stanley BA and Chiang H-L, Glucose induces rapid changes in the secretome of Saccharomyces cerevisiae. *Proteome Science* **12**:9-9 (2014).

42. Granucci N, Pinu FR, Han T-L and Villas-Boas SG, Can we predict the intracellular metabolic state of a cell based on extracellular metabolite data? *Molecular BioSystems* **11**:3297-3304 (2015).

43. Dhananjaya BL, Nataraju A, Raghavendra Gowda CD, Sharath BK and D'Souza CJ, Vanillic acid as a novel specific inhibitor of snake venom 5'-nucleotidase: a pharmacological tool in evaluating the role of the enzyme in snake envenomation. *Biochemistry Biokhimiia* **74**:1315-1319 (2009).

44. Dhananjaya BL, Nataraju A, Rajesh R, Raghavendra Gowda CD, Sharath BK, Vishwanath BS and D'Souza CJ, Anticoagulant effect of Naja naja venom 5'nucleotidase: demonstration through the use of novel specific inhibitor, vanillic acid. *Toxicon : official journal of the International Society on Toxinology* **48**:411-421 (2006).

45. Itoh A, Isoda K, Kondoh M, Kawase M, Kobayashi M, Tamesada M and Yagi K, Hepatoprotective effect of syringic acid and vanillic acid on concanavalin a-induced liver injury. *Biological & pharmaceutical bulletin* **32**:1215-1219 (2009).

46. Huang SM, Chuang HC, Wu CH and Yen GC, Cytoprotective effects of phenolic acids on methylglyoxal-induced apoptosis in Neuro-2A cells. *Molecular nutrition & food research* **52**:940-949 (2008).

47. Huang SM, Hsu CL, Chuang HC, Shih PH, Wu CH and Yen GC, Inhibitory effect of vanillic acid on methylglyoxal-mediated glycation in apoptotic Neuro-2A cells. *Neurotoxicology* **29**:1016-1022 (2008).

48. Garbe D, Cinnamic Acid, in *Ullmann's Encyclopedia of Industrial Chemistry*. Wiley-VCH Verlag GmbH & Co. KGaA (2000).

49. Fahlbusch K-G, Hammerschmidt F-J, Panten J, Pickenhagen W, Schatkowski D, Bauer K, Garbe D and Surburg H, Flavors and Fragrances, in *Ullmann's Encyclopedia of Industrial Chemistry*. Wiley-VCH Verlag GmbH & Co. KGaA (2000).

50. Eshkol N, Sendovski M, Bahalul M, Katz-Ezov T, Kashi Y and Fishman A, Production of 2-phenylethanol from L-phenylalanine by a stress tolerant Saccharomyces cerevisiae strain. *Journal of applied microbiology* **106**:534-542 (2009).

51. Butler MS, Natural products to drugs: natural product-derived compounds in clinical trials. *Natural product reports* **25**:475-516 (2008).

52. Katz L and Ashley GW, Translation and protein synthesis: macrolides. *Chemical reviews* **105**:499-528 (2005).

53. CH C, Vitamin B6 (Pyridoxine; Pyridoxal 5'-Phosphate). (2001).

54. Kark JA, Kale MP, Tarassoff PG, Woods M and Lessin LS, Inhibition of erythrocyte sickling in vitro by pyridoxal. *The Journal of clinical investigation* **62**:888-891 (1978).

55. Natta CL and Reynolds RD, Apparent vitamin B6 deficiency in sickle cell anemia. *The American journal of clinical nutrition* **40**:235-239 (1984).

56. Ryan-Harshman M and Aldoori W, Carpal tunnel syndrome and vitamin B6. *Canadian Family Physician* **53**:1161-1162 (2007).

Figure legends

Figure 1: Comparison of growth curve of *S. cerevisiae var boulardii* NCYC 3264 (*Sb*) and *S. cerevisiae* BY4742 (*Sc*) under normal laboratory and simulated *in vivo* conditions considering temperature and oxygen availability (Only the first 750 min were fitted due to the decline in optical density after this time in some cultures)

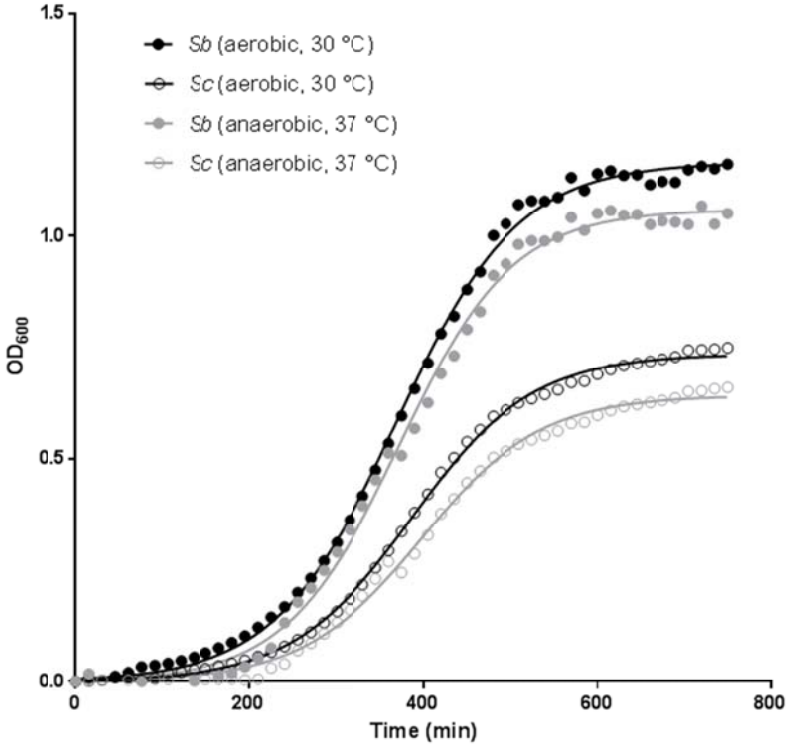
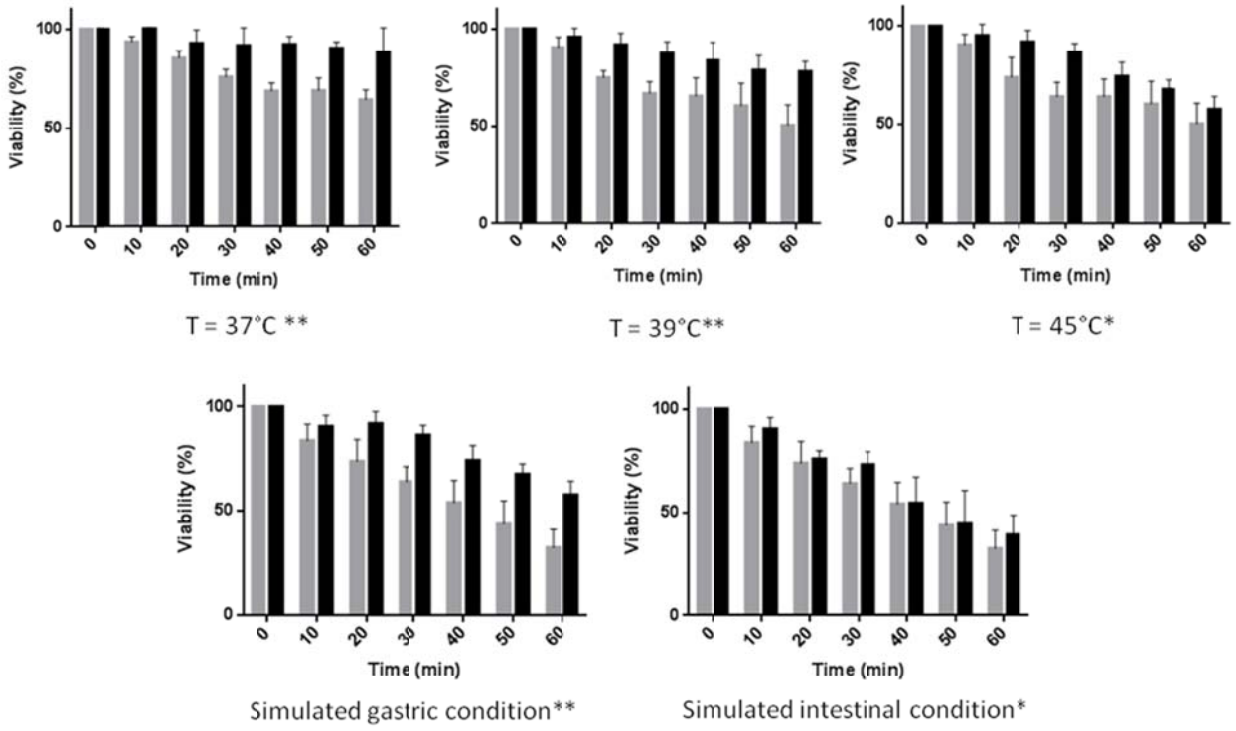


Figure 2: Percentage cell viability of *S. cerevisiae var. boulardii* NCYC 3264 (black) and *S. cerevisiae* BY4742 (grey) as a response to heat stress and tolerance to simulated gastric and intestinal conditions (T = Temperature).



Statistical difference indicated as * (p value < 0.05) and ** (p value < 0.01)

Figure 3: Spot assay for viability on agar media containing different concentrations of bile salts at different temperatures. *Sb – S. cerevisiae var boulardii* NCYC3264*; Sc – S. cerevisiae* BY4742

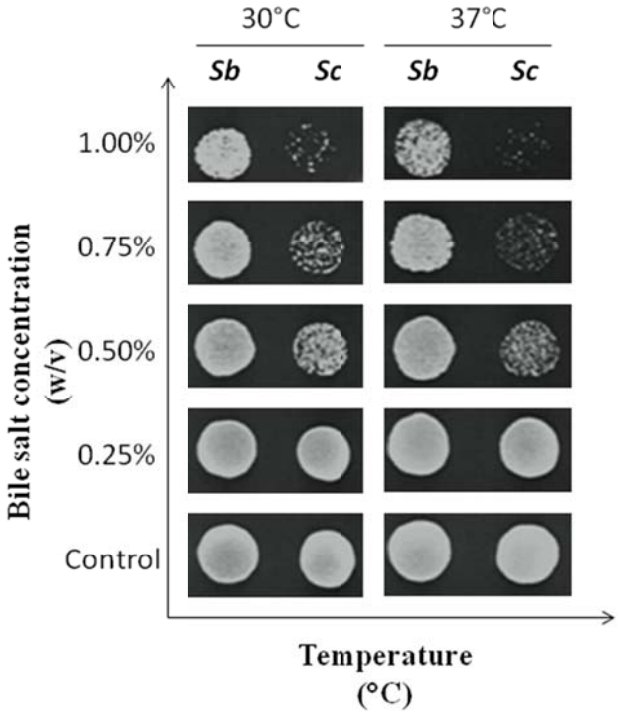
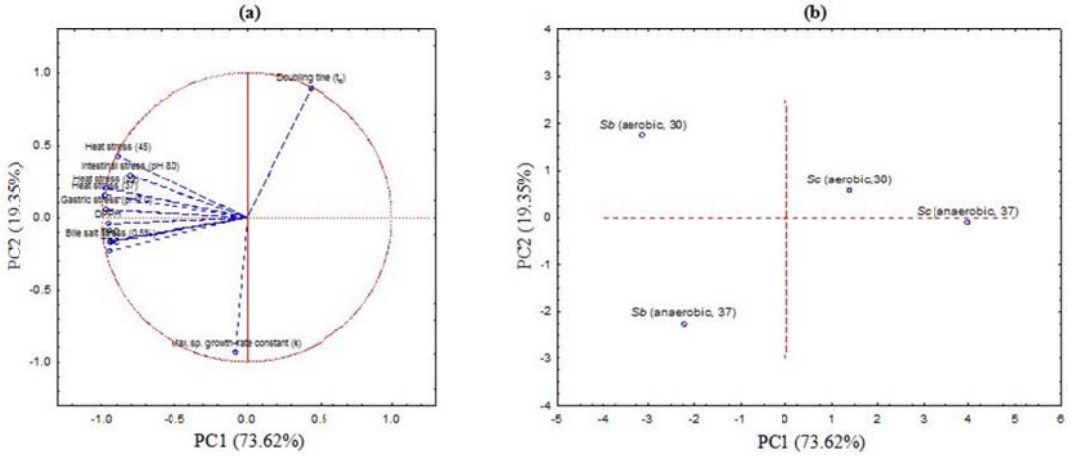


Figure 4: Projection of (a) growth kinetic, probiotic and antioxidant attributes as variables and (b) strains of *S. cerevisiae* var. *boulardii* NCYC 3264 (*Sb*) and *S. cerevisiae* BY4742 (*Sc*) under two different conditions (aerobic, 30 °C and anaerobic, 37 °C) as cases on the two-component plane.



Supplementary Figure S1: Scree plot of eigenvalues of components determining the selection of the first and second as the principal components (PC1 and PC2) showing the maximum possible variances.

Table1. Comparison of maximum specific growth rates (k), optimum doubling times (t_d) and maximum optical density (OD_{max}) of microwell cultures under different conditions.

Strain	**Laboratory conditions** *(Aerobic, 30 °C)*			**Simulated *in vivo* conditions** *(Anaerobic, 37 °C)*		
	k (min^{-1})	**t_d (min)**	**OD_{max}**	**k (min^{-1})**	**t_d (min)**	**OD_{max}**
S. cerevisiae var. boulardii (NCYC 3264)	0.0142 ± 0.0003	48.81	1.1660 ± 0.0066	0.0152 ± 0.0004	45.60	1.0590 ± 0.0072
S. cerevisiae S288C (BY4742)	0.0143 ± 0.0004	48.47	0.7349 ± 0.0056*	0.0145 ± 0.0005	48.80	0.6421 ± 0.0062*

Values of k and OD_{max} were determined using non-linear curve fitting (see Materials and Methods). Only the first 750 min were fitted due to the decline in optical density after this time in some cultures.

*Statistically significantly different ($p<0.05$)

Table 2. Comparison of antioxidant properties of *S. cerevisiae var.boulardii* (NCYC 3264) and *S. cerevisiae* S288C (BY4742).

Activity	*S. cerevisiae var.boulardii* (NCYC 3264)		*S. cerevisiae* S288C (BY4742)	
	Cell-free extract	Ethyl acetate fraction	Cell-free extract	Ethyl acetate fraction
TPC (mg GAE.g^{-1})	7.11 ± 0.30	7.05 ± 0.25	0.14 ± 0.05*	0.06 ± 0.08*
TFC (mg QE.g^{-1})	0.10 ± 0.04	0.11 ± 0.20	0.006 ± 0.004*	0.004 ± 0.006*
DPPH assay (% Inhibition)	33.16 ± 0.11	28.49 ± 0.20	5.28 ± 0.30*	3.62 ± 0.41*
TEAC (µM)	141.89 ± 0.22	144.86 ± 0.31	- [a]	- [a]

[a] No detectable activity

*Statistically significantly different ($p<0.05$) from *S. cerevisiae var.boulardii* (NCYC 3264)

TPC, total phenolic content; TFC, total flavonoid content; DPPH, scavenging activity of 1,1-diphenyl-2-picrylhydrazyl, TEAC, trolox equivalent antioxidant capacity assay. For assay conditions etc, see Materials and Methods.

Table 3. Component matrix of correlation coefficients of (a) variables and (b) cases on 2-component plane extracted from principal component analysis (PCA).

(a)

Variables	Component	
	1	2
Maximum sp. growth rate constant (k)	.084	-.935
Doubling time (t_d)	-.434	.895
Heat stress (37)	.981	.157
Heat stress (39)	.977	.208
Heat stress (45)	.891	.429
Gastric stress (pH 2.0)	.970	.061
Intestinal stress (pH 8.0)	.806	.298
Bile salt stress (0.5% w/v)	.941	-.171
Antioxidant capacity 1 (TPC)	.944	-.164
Antioxidant capacity 2 (TFC)	.951	-.227
Antioxidant capacity 3 (DPPH)	.960	-.042

TPC, total phenolic content; TFC, total flavonoid content; DPPH, scavenging activity of 1,1-diphenyl-2-picrylhydrazyl. For assay conditions etc, see Materials and Methods.

(b)

Cases	Component	
	1	2
S. cerevisiae var. boulardii NCYC 3264 (aerobic, 30°C)	30.13494	36.13039
S. cerevisiae BY4742 (aerobic, 30°C)	5.94069	4.11739
S. cerevisiae var. boulardii NCYC 3264 (anaerobic, 37°C)	15.34723	59.65203
S. cerevisiae BY4742 (anaerobic, 30°C)	48.57714	0.10019

Table 4. List of metabolites identified by GC-MS in the extracellular and intracellular fraction of (a) *S.cerevisiae var boulardii* NCYC 3264 and (b) *S. cerevisiae* BY4742 culture. R_t = retention time in min, MCF = Methyl chloroformate, m/z = mass to charge ratio of target ion in the spectrum

(a)

Peak no.	Extracellular fraction				Intracellular fraction			
	R_t (min)	**Metabolite**	**MCF derivatives**	**m/z (a.m.u)**	**R_t (min)**	**Metabolite**	**MCF derivatives**	**m/z (a.m.u)**
1.	8.902	Succinic acid	Dimethyl ester	146	8.915	Succinic acid	Dimethyl ester	146
2.	9.222	D-glucose	2,3,6-tri-*o*-methyl ester	222	10.786	L-alanine	*N*-methoxycarbonyl-ethyl ester	175
3.	9.427	1,3-dioxolane	2-methoxymethyl-2,4,5-trimethyl-ester	160	11.782	Hydroxylamine	-	173
4.	10.446	Phenyl ethyl alcohol (rose oil)	-	122	12.615	M-cymene	Methyl ester	190
5.	10.594	Amphetamine	*N*-methoxy carbonyl ester	193	13.992	L-leucylglycine	*N*-methoxycarbonyl-methyl ester	260
6.	10.937	2-pentenoic acid	4-methyl- ester	128	14.315	L-phenylisoquinoline	-	205
7.	11.477	Benzeneacetic acid (mephaneine)	4-methyl ester	150	14.757	L-proline	*N*-methoxycarbonyl-ester	257
8.	12.621	M-cymene	Methyl ester	190	14.773	D-proline,	*N*-methoxycarbonyl-ester	187
9.	13.095	Hydroxycinnamic acid	Methyl ester	164	14.888	Hydroxylamine	-	173
10.	13.992	L-leucylglycine	*N*-methoxycarbonyl-methyl ester	260	15.495	DL-aspartic acid	*N*-acetyl-dimethyl ester	203

11.	15.495	DL-aspartic acid	*N*-acetyl-dimethyl ester	203	15.809	Citric acid	Trimethyl ester	234
12.	10.594	Amphetamine	*N*-methoxy carbonyl ester	193	16.206	Pyruvic acid	Methyl ester	174
13.	15.805	Citric acid	Trimethyl ester	234	16.438	2,4-*bis*(1,1-dimethylethyl)-phenol (antioxidant no. 33)	-	206
14.	16.540	Cinnamic acid	Methyl ester	162	16.571	L-alanine	Methyl ester	317
15.	16.571	L-alanine	Methyl ester	317	17.764	Quinoline	Methyl ester	235
16.	16.654	Acetic acid	methyl ester	214	19.871	L-phenylalanine	Methyl ester	297
17.	17.503	2,4-dinitro-1-naphthalenol (golden yellow)	-	234	26.135	Fumaric acid	Dimethyl ester	245
18.	17.518	Quinoline	Methyl ester	235	29.613	Malic acid	Trimethyl ester	233
19.	17.929	Erythromycin	-	733				
20.	18.679	Vanillic acid	Methyl ester	182				
21.	19.871	L-phenylalanine	Methyl ester	297				
22.	23.890	Vitamin B6	Methyl acetate	295				

(b)

Peak no.	Extracellular fraction				Intracellular fraction			
	R_t (min)	Metabolite	MCF derivatives	m/z (a.m.u)	R_t (min)	Metabolite	MCF derivatives	m/z (a.m.u)
1.	12.102	D-glucose	Dimethyl ester	146	16.206	Pyruvic acid	Methyl ester	174
2.	15.805	Citric acid	Trimethyl ester	234	18.932	L-alanine	Dimethyl ester	116
3.	16.113	Lactic acid	Dimethyl ester	191	22.265	Valine	Dimethyl ester	144
4.	23.952	Succinic acid	tetramethyl ester	247	23.704	Ethanolamine	Dimethyl ester	174
5.	24.062	Glycerol	Trimethyl ester	218	23.986	Isoleucine/leucine	Dimethyl ester	158
6.	25.982	Fumaric acid	Dimethyl ester	245	24.068	Glycerol	Trimethyl ester	218
7.	27.624	Malic acid	Trimethyl ester	233	25.034	Glycine	Dimethyl ester	102
8.					25.1	Succinic acid	tetramethyl ester	247
9.					25.817	Uracil	Dimethyl ester	241
10.					26.135	Fumaric acid	Dimethyl ester	245
11.					26.243	Serine	Trimethyl ester	204
12.					26.992	Threonine	Trimethyl ester	218
13.					28.647	Homoserine	Trimethyl ester	218
14.					29.613	Malic acid	Trimethyl ester	233
15.					30.004	Erythritol	4-methyl ester	217
16.					30.092	Cytosine	Dimethyl ester	254
17.					30.501	Aspartate	Trimethyl ester	218

18.	31.906	A-ketoglutaric acid	Dimethyl ester	198
19.	32.903	Glutamate	Trimethyl ester	246
20.	33.102	Phenylalanine	Dimethyl ester	192
21.	33.419	Ribose	4-methyl ester	217
22.	33.902	Asparagine	Trimethyl ester	231
23.	35.264	2-aminoadipic acid	Trimethyl ester	260
24.	35.802	Orotic acid	Trimethyl ester	254
25.	36.051	Glycerol-1-phosphate	4-methyl ester	370
26.	36.452	Glutamine	Trimethyl ester	156
27.	36.742	N-acetyl-glutamic acid	Dimethyl ester	216
28.	37.312	Citric acid	4-methyl ester	273
29.	37.506	Ornithine	4-methyl ester	258
30.	39.311	Lysine	4-methyl ester	230
31.	39.842	Mannitol	Hexamethyl ester	307
32.	40.002	Tyrosine	Trimethyl ester	218
33.	42.946	Inositol	Hexamethyl ester	305
34.	43.005	Xylulose-5-phosphate	5-methyl ester	315
35.	46.821	Glucose-6-phosphate	Hexamethyl ester	387

Table S1. Eigenvalues for each component explaining the total variance displayed after principal component analysis.

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	8.098	73.619	73.619	8.098	73.619	73.619	7.866	71.513	71.513
2	2.128	19.349	92.968	2.128	19.349	92.968	2.360	21.455	92.968
3	.774	7.032	100.000						
4	3.739E-016	3.399E-015	100.000						
5	2.486E-016	2.260E-015	100.000						
6	9.181E-017	8.347E-016	100.000						
7	8.614E-017	7.830E-016	100.000						
8	1.653E-018	1.502E-017	100.000						
9	-1.134E-016	-1.031E-015	100.000						
10	-2.253E-016	-2.048E-015	100.000						
11	-4.340E-016	-3.946E-015	100.000						