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Short communications

Lipid droplet levels vary heterogeneously in response to simulated gastrointestinal stresses in different probiotic *Saccharomyces cerevisiae* strains

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ABSTRACT

To exert their therapeutic action, probiotic *Saccharomyces cerevisiae* strains must survive harsh digestive environments. Lipid droplets accumulate in cells which undergo stress-inducing situations, supposedly having a protective role. We assessed lipid droplet levels, either naturally accumulated or induced in response to digestive challenges, of probiotic strains *S. boulardii*, *S. cerevisiae* A-905, *S. cerevisiae* Sc47 and *S. cerevisiae* L11, and of non-probiotic strains *S. cerevisiae* BY4741 and *S. cerevisiae* BY4743. Strains 905 and Sc47 had lower and higher lipid droplet levels, respectively, when compared to the remaining strains, showing that higher accumulation

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of these neutral lipids is not a feature shared by all probiotic *Saccharomyces* strains. When submitted to simulated gastric or bile salts environments, lipid droplet levels increase in all tested probiotic strains, at least for one to the induced stresses, suggesting that lipid droplets participate in the protective mechanisms against gastrointestinal stresses in probiotic *Saccharomyces* yeasts.

1. Introduction

Lipid droplets (LD), the fat reservoirs of eukaryotic cells, are composed mostly of triacylglycerols (TAG) and sterol esters (SE), and are involved in many biological processes, such as inflammation, immune response, antigen presentation and interactions with pathogens (Saka & Valdivia, 2012). These intracellular organelles play a role in the lipid homeostasis and tend to increase when cells undergo stress situations, such as endoplasmic reticulum, oxidative and osmotic stresses (Khor, Shen, & Kraemer, 2013), protecting the cell against the effects of misfolded proteins and toxic lipids (Hapala, Marza, & Ferreira, 2011). In *Saccharomyces cerevisiae*, LD levels have been shown to increase when yeasts are subjected to temperature and secretory stresses (Fei, Wang, Fu, Bielby, & Yang, 2009; Gaspar et al., 2008; Hapala et al., 2011), drug treatment (Garaiová, Zambojová, Šimová, Griač, & Hapala, 2014) and to high saline concentrations and nitrogen starvation (Madeira et al., 2014).

Some *S. cerevisiae* strains have probiotic properties, which can provide health benefits to human [and animal] hosts when administered in adequate amounts (Vieira, Teixeira, & Martins, 2013). To date, *S. cerevisiae* var. *boulardii* (henceforth designed as *S. boulardii*) is the only probiotic yeast approved by the FDA for human consumption (Czerucka, Piche, & Rampal, 2007), although several *S. cerevisiae* strains have proven probiotic potential (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014; Kourelis et al., 2010; Martins et al., 2005; Palma et al., 2015; Perricone, Bevilacqua, Corbo, & Sinigaglia, 2014; Van der Aa Kühle, Skovgaard, & Jespersen, 2005) and some are commercialised as animal feed additives and veterinary probiotics (Ferraretto, Shaver, & Bertics, 2012; Pérez-Sotelo et al., 2005; Zanello et al., 2013). Probiotic *S. cerevisiae* strains are used as therapeutics against several types of diarrhoea, colitis and other gastrointestinal tract (GIT) malaises (Czerucka et al., 2007). To exert their probiotic potential, these yeasts must survive the harsh environments of the GIT, such as gastric acidic pH, bile salts and intestinal proteases (Fietto et al., 2004). It has been proposed that *S. boulardii* resistance to GIT milieus is related with overexpression of genes related to stress responses (Edwards-Ingram et al., 2007). However, the mechanisms responsible for probiotic *S. cerevisiae* survival in the GIT are still scarcely unknown. In this work, due to the cellular protective role of LD against several stress conditions, we sought to investigate if these organelles also play a protective role in probiotic *S. cerevisiae* yeasts when these are submitted to digestive challenges.

2. Materials and methods

2.1. Reagents, strains and growth media

All reagents, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

S. cerevisiae strains used in this work are listed in Table 1. Yeasts were manipulated as previously described (Douradinha et al., 2014; Madeira et al., 2014; Martins et al., 2005) and grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at either 37 °C (probiotics) or 30 °C (non-probiotics). Following overnight growth, yeasts were diluted to the desired working optical density at 600 nm (OD_{600}).

2.2. Doubling time

To determine yeast doubling time, cells were diluted to an OD_{600} of 0.2 and grown in YPD at the referred temperatures and incubated in a Bioscreen C spectrophotometer (Growth Curves, Piscataway, NJ, USA), according to manufacturer's instructions, during 28 h. The OD_{600} was measured every 15 min. All strains reached stationary phase by 24 h (Fig. S1). Doubling time was calculated based on the OD_{600} values of early to mid-log phase with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) (Table S1).

Table 1 – *Saccharomyces* strains used in this work.

Strains	Origin	Supplier	Reference
Probiotics			
S. boulardii 17	Floratil®	Merck SA (Rio de Janeiro, Brazil)	(Blehaut et al., 1989)
S. cerevisiae UFMG A-905	Cachaça[a]	UFMG[b] (Belo Horizonte, Brazil)	(Martins et al., 2005)
S. cerevisiae Sc47	Biosaf	Lesaffre Brazil (Penha, Brazil)	(Pérez-Sotelo et al., 2005)
S. cerevisiae L11	Procreatin7	Lesaffre Brazil (Penha, Brazil)	(Ferraretto et al., 2012)
Non-probiotics			
S. cerevisiae BY4741	–	Open Biosystems (Lafayette, CO)	(Winston et al., 1995)
S. cerevisiae BY4743	–	ATCC (Manassas, VA)	(Brachmann et al., 1998)

[a] Brazilian alcoholic spirit drink derived from sugarcane fermentation.
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2.3. *Lipid droplets quantification using liquid fluorescence recovery assay (LFR)*

LD were quantified by LFR as previously described (Bozaquel-Morais, Madeira, Maya-Monteiro, Masuda, & Montero-Lomeli, 2010). Briefly, cells were grown for 24 h, washed in distilled water and aliquoted to an OD_{600} of 5.0. Cells were subsequently fixed with 3.7% formaldehyde. Cells were washed and then diluted in reading medium, which contains 5 μM of the neutral lipid probe BODIPY 493/503 (Life Technologies, Grand Island, NY, USA) and 500 μM of fluorescence quencher KI. Absorbance at 600 nm (A_{600}) and fluorescence recovery by cell addition at 495/510 were read in a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). The cellular neutral lipid content (LD_{index}) was determined as the ratio fluorescence intensity/cellular density (A_{600}).

2.4. *Lipid droplets quantification using flow cytometry*

Yeast cells were fixed and labelled with BODIPY as described above. Samples were acquired on an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and excited with the 488 nm laser. The mean fluorescence intensity (MFI) was determined in the singlet population of yeast cells and data were analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA) and GraphPad Prism. The different strains tested varied in size, as shown by their respective forward scatter (FSC) parameter. LD content was determined by normalising each strain MFI with its respective size proportion to *S. cerevisiae* BY4741 (Fig. S2).

2.5. *Lipid droplets quantification using confocal fluorescence microscopy*

LD number and area were quantified by fluorescence microscopy as described elsewhere (Bozaquel-Morais et al., 2010; Madeira et al., 2014). Cells were fixed and treated with BODIPY as above and images acquired in an Axio Observer Z1 microscope (Zeiss, Göttingen, Germany). Images were processed with Zen 2012 software (Zeiss). LD number and area were quantified in 200 cells per strain using ImageJ software (NIH, Bethesda, MD, USA).

2.6. *Thin layer chromatography (TLC)*

Yeast cells were centrifuged at 1500 *g*, washed once with water, heat dried and weighed. Lipids were extracted of 320 μg of each yeast sample as previously described (Bourque & Titorenko, 2009; Madeira et al., 2014), allowed to dry, redissolved in chloroform and resolved by two-dimensional TLC (Madeira et al., 2014; Schmidt, Ploier, Koch, & Daum, 2013). Lipids were developed using iodine vapour and spots quantified by densitometry using Image Master TotalLab 1.1 (TotalLab Ltd, Newcastle Upon Tyne, UK).

2.7. *Yeast survival* in vivo

The levels of yeast survival *in vivo* were quantified as described elsewhere (Martins, Veloso, Arantes, & Nicoli, 2009). Briefly, 10^8 viable yeasts were administered orally to female BALB/c mice (8–10 weeks) daily, during 10 days. On the last day, faeces were collected, submitted to serial dilutions in sterile PBS and plated on Sabouraud dextrose agar (Difco, São Paulo, SP, Brazil) supplemented with chloramphenicol (200 mg/L), at the yeasts canonical temperatures. Colony-forming units (CFU) were assessed after 48–72 h (Fig. S3A).

Mice were provided by the Universidade Federal de Minas Gerais (UFMG) Animal House (Belo Horizonte, MG, Brazil). All procedures were done following the Brazilian College for Animal Experimentation requirements, which follow the European standards for animal experiments (EU Directive 2010/63/EU). The study was approved by the UFMG Ethics Committee in Animal Experimentation (CETEA/UFMG, protocol no. 197/2007).

2.8. *Simulated GIT stresses*

Simulated GIT stresses were performed as described elsewhere (Fietto et al., 2004). Briefly, yeast cells were grown overnight and diluted to a final OD_{600} of 0.3, either in YPD (control), YPD 0.1% pancreatin (Thermo Fisher, Waltham, MA, USA) and 0.5% NaCl pH 8.0 (intestinal stress) or YPD 0.1% mixture of primary and secondary bile salts (biliary stress). For gastric stress, yeasts were diluted to the same final OD_{600} in water 2% dextrose (control) and in gastric juice pH 1.5 (Thermo Fisher) 2% dextrose. Following 1 h incubation, samples were collected to assess their viability and LD levels. For the viability assays, serial dilutions of each of the aforementioned conditions were plated onto YPD agar plates, allowed to grow for at least 24 h and the number of CFU quantified (Fig. S3B). To determine lipid content, cells were washed once with water, fixed and labelled with BODIPY as before and analysed by flow cytometry as described above.

2.9. *Statistical analysis*

Statistical analyses were performed using GraphPad Prism 5.0. Results were considered significant if, upon applying Student's paired, two-tailed t-test, p values were inferior to 0.05.

3. Results

3.1. *LD levels accumulated heterogeneously in different* S. cerevisiae *strains*

It has been previously shown that the peak of accumulation of LD in *S. cerevisiae* strains occurs at early stationary phase, around 24 hours after inoculation (Bozaquel-Morais et al., 2010; Kurat et al., 2006; Zanghellini et al., 2008). First, we confirmed that all the tested yeasts do reach the early stationary phase around this time-point (Fig. S1), as their doubling times are different (Table S1). Subsequently, to determine if the probiotic *S. cerevisiae* strains accumulate higher level of LD than the laboratorial non-probiotic strain *S. cerevisiae* BY4741 (BY4741), we assessed the neutral lipid levels by LFR assay (Fig. 1A). Interestingly, the LD levels varied greatly between strains. We observed that veterinary probiotic *S. cerevisiae* Sc47 (Sc47) had the highest LD content while *S. cerevisiae* A-905 (905) showed the lowest LD levels. The other tested yeasts, *S. boulardii* 17 (Sb)

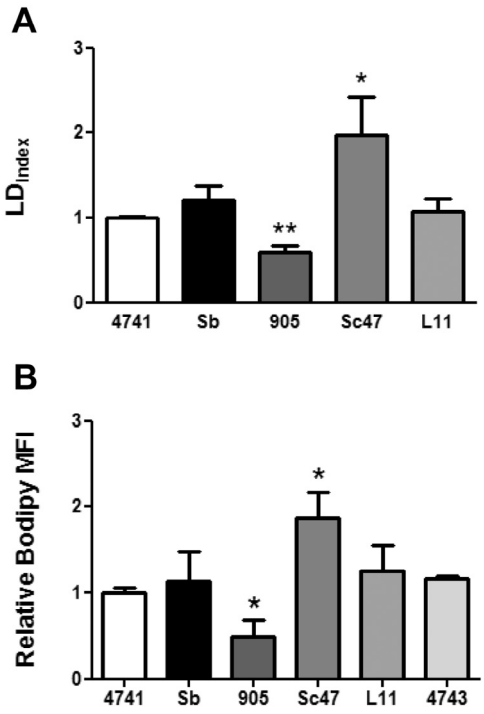

Fig. 1 – Lipid droplet levels varied among different *Saccharomyces* strains. LD content was quantified by (A) liquid recovery fluorescence and (B) flow cytometry. In both techniques, results are presented in proportion to the lipid content of BY4741. Strains 905 and Sc47 displayed lipid droplet levels significantly lower and higher, respectively, when compared to those of the haploid strain. No alterations in these organelle levels were observed in Sb, L11 and BY4743. Data shown derived from 3 independent experiments. For each strain, LD levels average of the 3 experiments ± standard deviation is plotted. * and ** correspond to $P < 0.05$ and $P < 0.01$, respectively.

and *S. cerevisiae* L11 (L11), did not present significant different LD levels when compared to the non-probiotic strain. To confirm these results, we quantified LD using flow cytometry (Fig. 1B), observing the same pattern in yeast lipid content. Since all probiotic strains tested were diploid while BY4741 was haploid, we decided to also quantify LD in a non-probiotic diploid strain, *S. cerevisiae* BY4743 (BY4743), to verify if their ploidy would be related with the LD level variations observed among the different strains studied. Assessment of BY4743 lipid content by flow cytometry did not differ significantly when compared to the haploid strain (Fig. 1B).

3.2. *LD numbers, areas and composition differed among the different yeast strains tested*

Next, using fluorescence microscopy, we quantified the number of LD per cell in each strain. Unsurprisingly, Sc47 and 905 had the highest and lowest numbers of cellular LD, respectively, among the tested strains (Fig. 2A). LD area did not vary between yeasts, except for Sc47, which showed a significantly larger area of these organelles per cell (Fig. 2B and C).

LD are mainly constituted of SE and TAG (Saka & Valdivia, 2012), so we decided to assess LD composition by thin layer chromatography in these strains. Levels of TAG and SE were found to be equally proportional within all tested yeasts, except for 905, which presented very low amounts of SE (Fig. 2D).

3.3. *LD level alterations in S. cerevisiae yeasts submitted to GIT stresses were strain-specific and differed according to the type of stress induced*

Our results showed that LD levels varied heterogeneously in different *S. cerevisiae* strains which had reached the stationary growth phase. We next addressed the question if LD levels would increase when these strains are submitted to gastrointestinal stresses. Our preliminary results in BALB/c mice showed that probiotic strains had a better colonic survival rate than BY4741, following oral administration (Fig. S3A). Also, while all yeasts survived and grew well in simulated intestinal conditions (data not shown), growth levels during simulated gastric and bile salts conditions indicated very different survival rates between the several studied strains (Fig. S3B). We also assessed the LD levels by flow cytometry during simulated GIT stresses to confirm if they would increase, when compared with the respective untreated control. As expected, no variation of LD levels was observed when the strains were subjected to intestinal conditions (data not shown). However, the neutral lipid content increased in all strains after enduring the gastric environment, except for Sb and BY4743 (Fig. 3A). The presence of bile salts caused LD levels to remain unaltered in 905 and BY4743 but increased in the remaining tested strains (Fig. 3B).

4. Discussion

Probiotic microorganisms must survive the GIT adverse conditions so they can exert their therapeutic effect in the colon. Although resistance to GIT challenges is one of the first conditions tested in potential probiotic *Saccharomyces* strains (Palma et al., 2015), very few studies have so far investigated which potential mechanisms are responsible for the yeasts survival in such environments (Cascio et al., 2013; Sant'Ana et al., 2009; Edwards-Ingram et al., 2007). As mentioned before, it has been shown that LD levels increased in *S. cerevisiae* undergoing situations of cellular stress (Fei et al., 2009; Garaiová et al., 2014; Gaspar et al., 2008; Hapala et al., 2011; Madeira et al., 2014). However, no study has yet focused on LD behaviour of *S. cerevisiae* strains submitted to GIT harsh environments and the potential contribution of these organelles in yeast survival in such aggressive conditions. In this work, we assessed the levels of LD in probiotic and non-probiotic *S. cerevisiae* strains, either when they reached stationary phase or following simulated GIT harsh environment treatments. To the best of our knowledge, this is the first time LD are studied in probiotic *S. cerevisiae* strains.

Our first assumption was that *S. cerevisiae* strains with probiotic potential naturally accumulate higher levels of neutral lipids, which would help them to endure the severe GIT milieus. However, our results showed that LD levels and numbers were heterogeneous among probiotic and non-probiotic strains

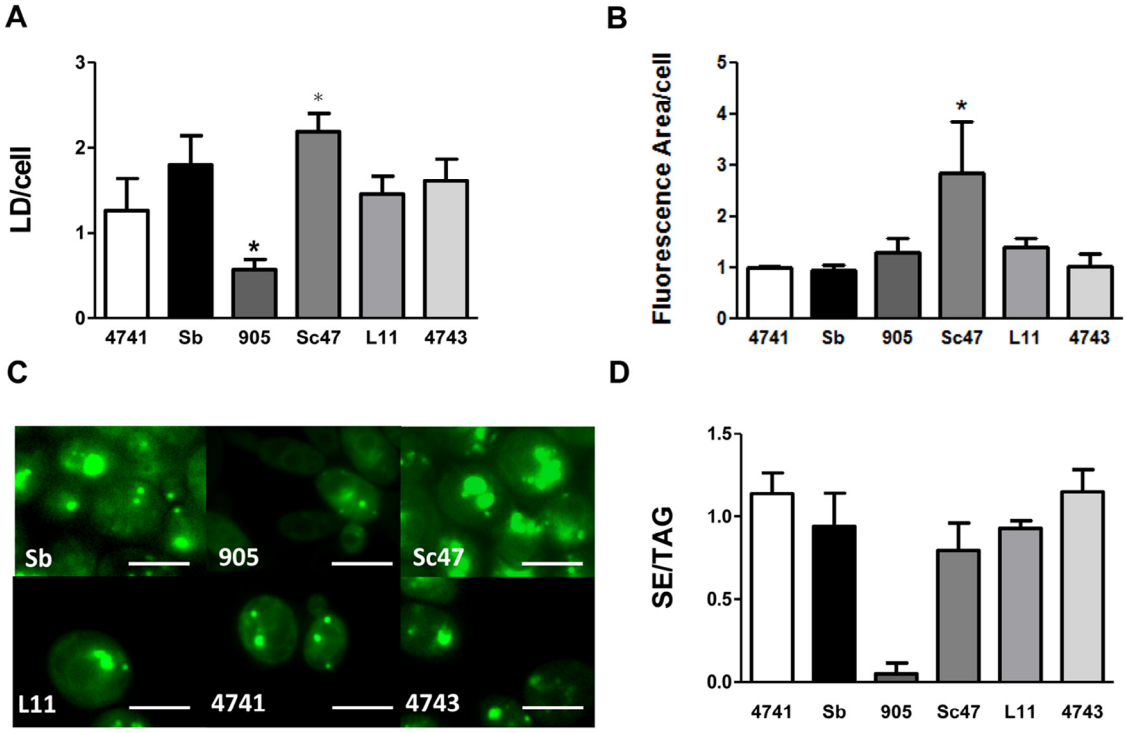

Fig. 2 – Lipid droplet numbers, areas and composition differed between the tested *Saccharomyces* strains. (A) Number of lipid droplets per yeast was determined by fluorescence microscopy and reflected what was observed above for the neutral lipids content, with similar variations for 905 and Sc47 when compared with BY4741. Sb, L11 and BY4743 displayed lipid droplet numbers per cell analogous to those of BY4741. (B) Area of lipid droplets was also quantified by fluorescence microscopy. On average, the area of these organelles did not differ in the tested strains, except for Sc47, which possessed larger lipid droplets. Values were normalised based on BY4741 lipid droplets area per cell (C) Fluorescence pictures of neutral lipids labelled with BODIPY (green) in the *Saccharomyces* strains studied. Bar, 10 μm. (D) Lipid droplet composition as determined by thin layer chromatography. Levels of triacylglycerols (TAG) and sterols (SE) were equally proportional within all tested yeasts, exception made for 905, which contained very low amounts of SE. Data shown derived from 3 independent experiments. For each strain, the average of (A) LD numbers, (B) LD areas and (D) ratio SE/TAG of the 3 experiments ± standard deviation is plotted. * corresponds to $P < 0.05$.

(Figs. 1 and 2A–C), suggesting that probiotic *S. cerevisiae* strains resistance to GIT harsh environments is unrelated with their initial neutral lipid accumulation levels. Interestingly, we observed that one of the studied probiotic strains, 905, possesses very low amounts of SE (Fig. 2D), which may account for the lower LD number and levels in this strain (Figs. 1 and 2A–C). LD composed mostly of TAG have been observed in *S. cerevisiae* W303 (W303) mutants unable to produce SE (Czabany et al., 2008). Since TAG occupies most of the LD inner core, it may explain why LD in 905, despite their low levels, did not exhibit smaller areas when matched to those of BY4741. Also, of all tested strains, 905 was the most resistant to gastric and bile salts simulated stresses (Fig. S3B). Repression of SE synthesis is related with resistance to high saline concentrations and oxidative stress in BY4741 (Montañés, Pascual-Ahuir, & Proft, 2011). In an acidic milieu, the expression of sterol esterase, an enzyme involved in SE biosynthesis, is downregulated in Sb (Cascio et al., 2013), a mechanism which probably contributes to its gastric stress resistance (Fig. S3B). Correspondingly, 905's natural inability to synthetise SE might render this yeast more resistance to the harsh GIT environments. Sc47 is a veterinary probiotic and feed additive used widely for fattening of pigs, cattle, rabbits and lambs (FEEDAP, 2012). Its effectiveness as a veterinary fattening agent is justified by its high lipid levels (Figs. 1 and 2A–C). L11 is another veterinary probiotic, usually recommended to increase milk quality and quantity in lactating animals (Ferraretto et al., 2012), with lipid levels similar to those of Sb and BY4741 (Figs. 1 and 2A–C). Both Sc47 and L11 survived poorly in simulated gastric and bile salts conditions (Fig. S3B), regardless of their lipid content levels. Animals have milder gastric pH and bile salts concentrations than humans (Martinez, Augsburger, Johnston, & Warren, 2002) and the conditions used in this work mimic those of the human GIT (Fietto et al., 2004), which explains why these probiotic strains are, nevertheless, effective in veterinary applications. Furthermore, our tests were done using yeasts in solution, while these animal probiotics are supplied to animals as dried particles (FEEDAP, 2012; Ferraretto et al., 2012), in which the outer layers of yeast protect the inner core from the aggressive GIT conditions, a feature similar to encapsulation in Sb (Graff, Chaumeil, Boy, Lai-Kuen, & Charrueau, 2008). Our preliminary results showed that these veterinary probiotic strains, when administered to BALB/c mice, had a higher survival rate than BY4741 (Fig. S3A). Also, their low doubling time (Table S1) would allow a faster recovery in

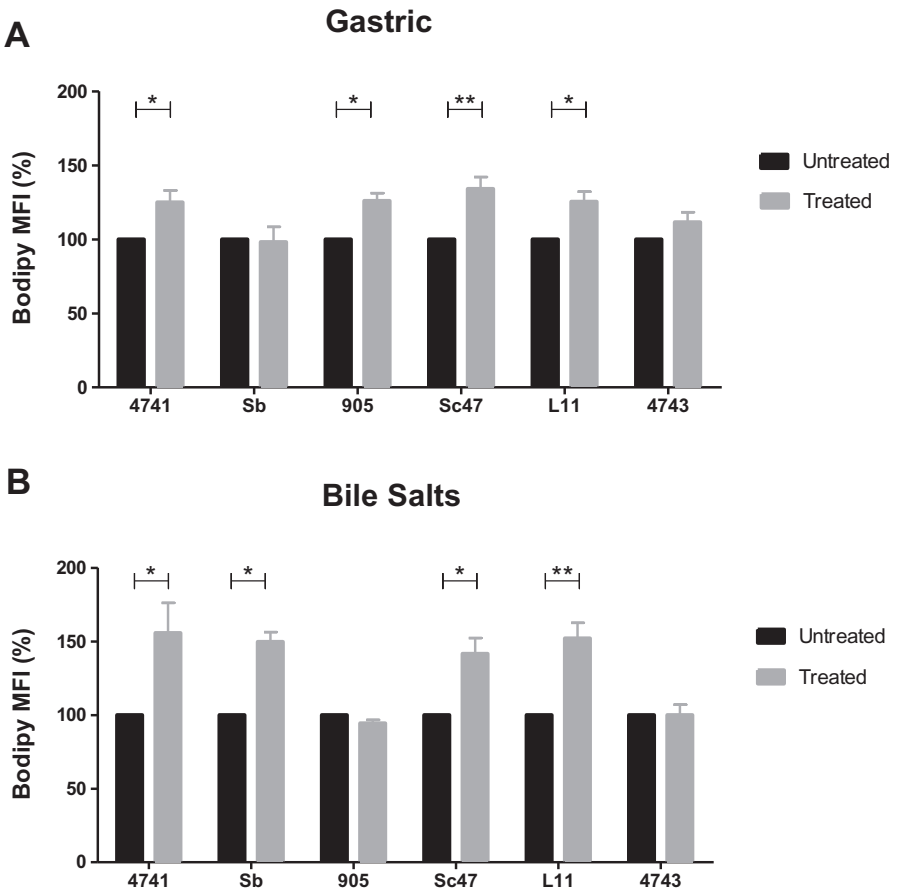

Fig. 3 – Influence of simulated (A) gastric and (B) bile salts stresses in *Saccharomyces* strains LD levels. Yeast neutral lipid levels content was measured by cytometry after 1 h of treatment with gastric juice or bile salts and matched to corresponding untreated controls. All probiotic yeasts had higher levels of LD in at least one harsh condition studied, suggesting these organelles played a protective role when these strains are submitted to GIT stresses. Likewise, neutral lipids increased in BY4741 in both low pH and bile salts conditions indicating a similar type of protection as for the probiotic strains. On the other hand, BY4743 LD levels remained unchanged in both stresses, suggesting that this strain in particular possessed other mechanisms to resist the GIT harsh environments. Data shown derived from 3 independent experiments. For each strain, the average of MFI for both untreated and stress-submitted conditions of the 3 experiments ± standard deviation is plotted.* and ** correspond to $P < 0.05$ and $P < 0.01$, respectively.

the GIT than the other probiotic strains used in this work, contributing to their effective probiotic action.

As previously shown, neutral lipid levels tend to increase in *S. cerevisiae* strains undergoing stressful events (Fei et al., 2009; Garaiová et al., 2014; Gaspar et al., 2008; Hapala et al., 2011; Khor et al., 2013; Madeira et al., 2014) and, since initial LD levels in probiotic yeasts did not relate with their resistance to GIT challenges, we decided to quantify these neutral lipids in the studied strains after submitting them to GIT simulated stresses. We observed that Sb and BY4743 LD levels remained unchanged following gastric acidic pH treatment (Fig. 3A). Sb possesses an efficient H^+-efflux system, which allows this yeast to maintain its intracellular pH at neutral levels and contribute to its survival in acidic conditions (Sant'Ana et al., 2009). Plus, Sb differentially regulates genes related with stress response in low pH conditions (Cascio et al., 2013). These combined mechanisms seem to be sufficient to promote Sb survival in gastric conditions, suggesting that increasing LD levels in this situation would be redundant and lead to an unrequired consumption of cellular metabolic energy. Our results lead to believe that BY4743 survival in acidic pH levels relies upon similar mechanisms. It is currently unknown if the other *S. cerevisiae* strains tested share these protective mechanisms. A laboratorial strain, W303, displayed low H^+-ATPase activity, rendering it much more sensitive to acidic pH levels (Sant'Ana et al., 2009). We assume that the remaining strains used in this work have a weak proton efflux capacity similar to W303, although that remains to be confirmed. The observed increase in LD levels in these *S. cerevisiae* strains following gastric juice treatment (Fig. 3A) would compensate as a protective mechanism and allow them to resist acidic stress.

Resistance to bile salts induced stress also varied in the tested strains (Fig. 3B and Fig. S3B). Thus, it is not surprising that exposure to a biliary environment would lead the least resistant strains into stress and consequently to the increase observed in their neutral lipid levels (Fig. 3B), suggesting they use an LD-based protective mechanism when submitted to this particular GIT stress. Both 905 and BY4743 strains did not show

an increment in their LD levels, indicating they depend on other mechanisms to survive bile salts action (Fig. 3B and Fig. S3B).

Our work addressed, for the first time, the issue of LD in probiotic microorganisms and their participation in protection against GIT stresses, contributing to our understanding of the mechanisms used by *S. cerevisiae* to survive the challenges suffered while transiting the gut mucosa. Currently, there are no reliable genetic engineering systems that efficiently knockout genes in diploid, industrial yeasts. For these strains, genetic manipulation is highly complex, although some groundbreaking results have been recently observed (Stovicek, Borodina, & Forster, 2015). Such systems would allow us to determine the role of specific enzymes involved in neutral lipid biosynthesis by disrupting the genes which are responsible for their expression in industrial *S. cerevisiae* strains, such as those used in this work. Also, lipid metabolism inhibitors have antifungal properties (Fernandes, 1992), which would induce *per se* stress in yeasts and could mask the effects caused by other types of stressful conditions, such as digestive challenges. Development of inhibitors of specific neutral lipids, that would discriminate the contribution of each individual lipid and would not be toxic to yeasts, would help unravel further the specific mechanisms of resistance to the GIT harsh environments used by each particular strain and how that influences its probiotic potential.

5. Conclusions

LD levels increased in *S. cerevisiae* strains when they were subjected to simulated gastric and bile salts environments. Plausibly, the increase in these organelle levels acted as a protective mechanism against these GIT stresses. All probiotic strains showed an increase in LD levels when submitted to at least one of the simulated GIT stresses, suggesting these organelles contribute to their survival and, consequently, to their probiotic effect. Also, in the tested conditions, proliferation of LD levels was not always observed, strengthening the idea that the yeasts used in this study possess other defensive mechanisms against stress, e.g., Sb proton-efflux protecting from low pH levels.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.12.013.

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