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Title: Influence of freezing temperatures prior freeze-drying on the viability of yeasts and lactic acid bacteria isolated from wine.

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Running title: Freezing effect on survival of wine microorganism

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Abstract

Aims: To determine the effect of three different freezing temperatures on survival rates of wine yeasts and lactic acid bacteria (LAB) after freeze-drying**.**

Methods and Results: Yeasts were grown in GPYD broth, *Lactobacillus, Pediococcus* and *Leuconostoc* strains in MRS broth and *Oenococcus oeni* in MLO broth to mid stationary phase. Cells were recovered and concentrated in appropriate lyoprotectants. Aliquots of each strain were frozen at -20, -80 and -196ºC before vacuum drying. Viable cells counts were performed before freezing and after freeze-drying and survival rates calculated. Yeast and bacteria survivals were affected differently by temperatures of freezing. The highest survivals rates were obtained at -20 ºC and -80 ºC for yeasts, whereas for LAB they were at -196ºC, although no significant differences among the three temperatures were obtained in this case. Wide differences in survival rates were recorded among freeze-dried yeasts, while these differences were much less drastic in the case of LAB species. *Pichia membranifaciens*, *Starmerella bacillaris* and *Metschnikowia pulcherrima* among yeasts and *Lactobacillus paracasei*, *Pediococcus parvulus* and *Lactobacillus mali*, among LAB, were the most tolerant species to freeze-drying.

Conclusions: -20ºC is the temperature that ensures the highest viability after the lyophilization process for yeast, whereas -196ºC guarantees the best results for LAB.

Significance and Impact of the Study: This study shows that freezing temperature used to lyophilize cells is a crucial factor for ensuring a good survival of wine yeast and LAB. These results have important practical applications, not only for appropriately preserve microorganisms but also for improving starter production processes.

Keywords : freeze-drying, yeast, lactic acid bacteria, freezing, lyophilization, survival, wine.

Introduction

Yeast and lactic acid bacteria are involved in a great number of traditional European, African and Asiatic food fermentations that result in products as basic as bread, sausages, dairy products, sauerkraut, beverages, or as exotic as gari, idli, ogi, etc. (Caplice and Fitzgerald 1999; Holzapfel 2002). Preservation of microorganisms responsible for these fermentations is of great interest at both domestic and industrial levels. Careful preservation of microorganism is imperative for future research, teaching and industrial applications (Prakash *et al.* 2013). Lyophilization (or freeze-drying) is the preferred long-term preservation method used by microbial resource centres and by industries producing bacterial starters (Høier *et al.* 1999; Krieger 2009; Prakash *et al.* 2013), due to ease of transportation and use, to the low cost of maintenance, and to the high rate of cell survival over long periods of time (Miyamoto-Shinohara *et al.* 2006; Morgan *et al.* 2006; Prakash *et al.* 2013). Freeze-drying is a form of preservation based on a cold-drying process which consists in the dehydration of a substance by sublimation and involves three phases: freezing, primary drying (sublimation) and secondary drying (desorption) (Kumar *et al.* 2011). Freeze-drying ~~Lyophilization~~ is considered the most appropriate method for preservation of microorganisms. Freeze-drying is a very complex physical process in which cell survival is affected by many physico-chemical and biological parameters. Physico-chemical factors such as cell growth conditions, type of lyoprotectant, freezing, sublimation and thawing temperatures, degree of dehydration achieved, reconstitution medium, and time and storage and rehydration conditions have been described to have great influence on the survival of microorganisms (Donev *et al.* 1995; Dumont *et al.* 2004; Zhao and Zhang 2005; Nakamura *et al.* 2009; Peiren *et al.* 2015). Also, biological factors, as type of microorganism, initial cell concentration, age of the cells, and presence of compatible solutes in cell cytoplasm that can affect freeze-dried cell survival (Donev *et*

al. 1995; Miyamoto-Shinohara *et al.* 2000). Freeze-drying implies always a fall of viable cells (Tymczyszyn *et al.* 2007), since it affects greatly cell compounds, structures and properties, especially those related to cell membranes. The main causes of losing viability after freeze-drying are, probably, ice crystal formation, high osmolarity due to the increase of internal solute concentration, and macromolecule denaturation induced by water removal (Pehkonen *et al.* 2008). Although freezing itself has not a lethal effect for cells, it can induce physical stress that can injure a part of these cells, thus decreasing the proportion of viable cells (Pehkonen *et al.* 2008). If freezing is extremely slow, intracellular water can flow to the outer environment by osmosis and create extracellular crystals, thus causing extracellular water removing and solute concentration increase that lead to an osmotic imbalance. Conversely, if freezing is too fast, cells can not lose water fast enough to maintain the balance, so intracellular ice crystals can appear thus producing damaging or even lethal effects (Seki *et al.* 2009).

For these reasons, not all microorganism can be successfully preserved by this method, thus satisfactory results are obtained for many bacteria, yeast and sporulating fungi, but are not for non-sporulating fungi, some yeast species (*Lipomyces*, *Leucosporidium*, *Brettanomyces*, *Dekkera*, *Bullera*, and *Sporobolomyces*) and certain bacteria (*Aquaspirillum serpens*, *Clostridium botulinum*, *Helicobacter pylori*, etc.) (Prakash *et al.* 2013).

The aim of this work was to evaluate the influence of different initial freezing temperatures prior sublimation on the viability of wine-isolated microorganisms subjected to freeze-drying. Improving preservation of these important industrial microorganisms is of paramount interest for industries devoted to starter culture production.

Materials and methods

Microorganisms

Microorganisms used for experiments are described in Table 1. Strains are representative of the species currently found during vinification and were obtained from the Spanish Type Culture Collection (CECT) and the private wine microorganism culture collection ENOLAB (University of Valencia).

Biomass production

Yeast strains were grown in Glucose Peptone Yeast extract medium (GPY). The composition per litre was as follows: 20 g glucose (Panreac); 10 g acid casein peptone H (Pronadisa); yeast extract 5 g (Pronadisa) and agar 20 g (Pronadisa). The pH was adjusted to 5.5.

Lactobacillus, *Pediococcus* and *Leuconostoc* species were grown in de Man, Rogosa and Sharp medium (MRS) (Scharlab) supplemented with L-Cysteine (0.5 g l^{-1}) (Sigma) and *Oenococcus oeni* Enolab 4168 was grown in Medium for *Leuconostoc oenos* medium (MLO), pH 4.8 (Zúñiga *et al.* 1993). One hundred millilitres cultures were incubated at 28ºC until mid-stationary phase; yeasts cultures were grown under shaking conditions 160 rpm in a HT Infors AG rotatory shaker, whereas bacteria biomass was produced without agitation. Yeast and bacteria were harvested by aseptic centrifugation at 6000 rpm (6842 ***g*)** for 15 minutes at 4ºC in a Multifuge 1 S-R centrifuge (Heraeus). The yeast pellet was washed with 100 ml of 0.9% NaCl (Panreac) solution, centrifuged again at the same conditions and resuspended in a lyoprotectant solution consisting in 2 ml of skimmed milk powder (Oxoid) and 1 ml of 15% glucose (Panreac) solution. Bacterial cells, after centrifugation from the culture media, were washed with the same volume of glutamic acid (0.067 mol l^{-1}) and centrifuged under the same conditions. A concentrated

cell suspension was prepared by resuspending the cell pellet with 3 ml of glutamic acid (0.067 mol l^{-1}).

Freeze-drying and rehydration protocols

Volumes of 300 µl of cell suspensions were distributed into 0.5 mm diameter sterile glass tubes and, after 10 minutes at room temperature, they were subjected to different freezing treatments: -20 ºC for one hour, -80 ºC for one hour and -196 ºC (by immersion in liquid N_2) for one minute. Immediately after the treatments, cells were freeze-dried in a Virtis lyophiliser operating at a 15.9 millitorr vacuum pressure for 18 hours. After freeze-drying vials were sealed under a vacuum pressure of 1.7 millitors and then, stored at 4 ºC for a week. After this storage period, cells were rehydrated and homogenized with 300 µl of the appropriate culture media: GPY for yeasts, MRS for *Lactobacillus*, *Pediococcus* and *Leuconostoc* species and MLO for *O. oeni*. Three independent replicates of each strain were done.

Survival rate calculation

Viable cell counts were carried out on GPY, MRS or MLO plates, depending of the type of microorganism. Concentrated cell suspensions, taken just before freezing and after rehydration, were serially diluted and spread on plates. Plates were incubated at 28°C for 3-4 days and then the number of colony forming units per millilitre (CFU ml^{-1}) was determined. Survival percentage was calculated as the percentage of viable cells after freeze-drying relative to viable cells before freeze-drying. Survival assays were carried out in triplicate.

Statistical analysis

Statistical analyses were performed by using the JMP Pro 12 Statistical Software package (SAS Institute Cary NC, USA). Generally, the independent variable (survival rates) distribution was assessed for normality using the Shapiro-Wilk W Test. The homoscedasticity was tested using the Levene's test. When any of them failed, a variable transformation was performed in order to improve normality or homogenize the variances. As neither normality nor homoscedasticity improved, non-parametric Kruskal-Wallis rank sum test was used. Type of microorganism (yeast or bacteria), strain and freezing treatment were considered as dependent variables. Nonparametric comparisons for each pair using Wilcoxon Method were used to investigate the relationship between the different species. Statistical significance was judged at level P = 95%.

~~Existence of significant differences in survival relative to freezing temperatures was determined by Kruskal-Wallis ANOVA one way non parametric test, and considering different variables as factors: type of microorganism (yeast or bacteria, freezing temperature, yeast or LAB species, etc.). The SPSS Statistics (IBM) software version 22 was used. Statistical significance was judged at level P < 0.05.~~

Results

The values of survival of yeasts and LABs obtained from the three different freeze-drying processes are shown in Tables 2 and 3.

We found important differences in the response to freeze-drying between the yeast and LAB groups. Yeasts were more sensitive to freezing than LABs. Considering the three temperatures tested, average survival values were 5.6% for yeast and 21% for bacteria. The *p* value obtained after Kruskal Wallis ANOVA one way test of data showed that the null hypothesis that there were not differences in survival between the two groups of microorganisms was not supported ($p = 0.000$) (Figure 1). Yeast and bacteria survivals

were affected differently by the different temperatures of freezing. In the case of yeasts, higher survivals were obtained at -20ºC (average 9.5%) and at -80ºC (average 7.25%) than at -196 ºC (average 0.4%) (Table 2, Figure 2). On the contrary, survival at -20, -80 and -196ºC did not differ too much for LAB, (averages 21, 18 and 23%, respectively) (Table 3, Figure 3). Statistical analysis of yeast survival data showed no significant differences between at -20ºC and -80ºC (p = 1.000). However, there are statistical differences between -20°C and -196°C (p = 0.001) and between -80°C and -196°C (p = 0.014). In the case of bacteria, no significant differences in survival were found among the three temperatures tested (p = 0.695) (Figure 2).

As can be observed in Table 2, survival rates varied widely among individual yeast strains; values ranged between 38 and 0.00031%, and extreme values corresponded to *Pichia membranifaciens* frozen at -20 ºC and to *Saccharomyces cerevisiae* frozen at -196 ºC. Among yeasts, the highest survival rates were obtained at -20 ºC and the lowest at -196 ºC. Average survival rates of each yeast showed that the most resistant yeasts to freeze-drying (regardless of the temperature used) were *P. membranifaciens*, *Starmerella bacillaris* and *Metschnikowia pulcherrima*, whose survival exceeded 16%, whereas the rest did not overcome 5%. *Wickerhamomyces anomalus*, *S. cerevisiae*, *Dekkera bruxellensis*, *Schizosaccharomyces pombe* and *Issatchenkia occidentalis* strains showed much lower survival rates compared with the rest of yeasts, regardless of the freezing temperature used. Considering averages of survival percentages at the three temperatures as variable, and yeast strain as factor a boxplot was plotted (Figure 4). The null hypothesis - i.e. there do not exist differences in survival averages at the three temperatures among species - was not supported. Thus, significant differences in survival percentages have been recorded for the couples *D. bruxellensis* and *Hanseniaspora uvarum* (p= 0.023), *D.*

bruxellensis and *Torulaspora delbrueckii* (P= 0.008), *D. bruxellensis* and *M. pulcherrima* (*p*= 0.002), *D. bruxellensis* and *S. bacillaris* (P= 0.001), and *D. bruxellensis* and *P. membranifaciens* (*p*= 0.000); also for the couples *S. cerevisiae* and *T. delbrueckii* (*p*= 0.023), *S. cerevisiae* and *M. pulcherrima* (*p*= 0.005), *S. cerevisiae* and *S. bacillaris* (*p*= 0.002), and *S. cerevisiae* and *P. membranifaciens* (*p*= 0.001), *Issatchenkia occidentalis* and *M. pulcherrima* (P= 0.020), *I. occidentalis* and *S. bacillaris* (*p*= 0.009), *I. occidentalis* and *P. membranifaciens* (*p*= 0.005), *Schizosaccharomyces pombe* and *M. pulcherrima* (*p*= 0.043), *S. pombe* and *S. bacillaris* (P= 0.020), *S. pombe* and *P. membranifaciens* (*p*= 0.013), *Wickerhamomyces anomalus* and *S. bacillaris* (*p*= 0.043), and, finally, *W. anomalus* and *P. membranifaciens* (*p*= 0.028).

The null hypothesis that considers that yeast survival was affected in the same way for the different freezing temperatures could not be supported. Thus, the *P. membranifaciens*, *S. bacillaris*, *M. pulcherrima, W. anomalus, I. orientalis* and *S. cerevisiae* survivals were significantly different at -20°C and -196°C and at -80°C and -196°C, but no at -20°C and -80°C. For this group of strains is indistinct that freezing took place at -80 or -20ºC, as there are not significant differences between the survival observed at these temperatures. As for the rest of yeasts, the temperature of -196ºC was that provided lower survival values. Significant differences in *T. delbrueckii,* and *H. uvarum* survival rates were found among the three freezing temperatures tested, higher survivals rates were recorded at -20°C, while the lowest values were obtained at -196ºC. Significant differences in survivals rates of *I. occidentalis* were found between -20°C and -80°C and between -20°C and -196°C, but not between -80°C and -196°C, whereas *S. pombe* survivals were

significantly different only between -20ºC and -80ºC. In these two yeasts, freezing at -20°C ensured the highest survival rates, as for the rest. Finally, statistical significant differences were found for *D. bruxellensis* survival rates between -20°C and -80°C and between -80°C and -196°C, but not between -20°C and -196°C. The highest survival rate for this yeast was achieved at -80°C.

LAB survival rates after freeze-drying ranged between 52 and 1.4% (Table 3, Figure 5). The highest value corresponded to *Lactobacillus paracasei* frozen at -196ºC, whereas the lowest one to *Lactobacillus brevis* frozen at -80ºC. In general, the highest survival rates were obtained at -196ºC, however, values corresponding to -20ºC and -80ºC were only slightly lower; in fact, percentages of survival at these two temperatures were 90 and 78% of that observed at -196ºC, respectively. The most sensitive bacteria to freezing at -20 and -80ºC was *Lact. brevis*, but at -196ºC was *O. oeni*.

Considering the average of the survival rates of each freeze-dried bacteria at the three freezing temperatures used, a plot from Kruskal Wallis ANOVA one way analysis was built considering strain as the factor (Figure 5). Kruskal Wallis statistical test showed that significant differences in survival rates existed among bacteria (p= 0.000). Significant differences were found between *Lact. brevis* and *Lactparacasei* (p= 0.000), *Lact. brevis* and *Lact. mali* (p= 0.002), and *Lact. brevis* and *Ped. parvulus* (p= 0.000), Also, the survival rates of the next couples were significantly different: *Lact. plantarum* and *Lact. mali*, (p=0.033), *Lact. plantarum* and *Ped. parvulus* (p= 0.002), *Lact. plantarum* and *Lact. paracasei* (p= 0.000), *Leuc. mesenteroides* and *Ped. parvulus* (p= 0.006), *Leuc. mesenteroides* and *Lact. paracasei* (p= 0.001), and *Lact. paracasei and O. oeni* (p=0.016). Differences between the rest of the couples were not significant. The bacteria showing the highest survival rates, considering the averages of survival at all freezing

temperatures, were *Lact. paracasei, Ped. parvulus and Lact. mali* (Table 3, Figure 5). It can be deduced from Figure 5 that bacteria more tolerant to freeze-drying, whatever temperature used for freezing, were *Lact. paracasei*, *Ped. parvulus,* and *Lact. mali,* whereas *Lact. brevis, Lact. plantarum* and *Leuc. mesenteroides* were the less tolerant. No significant differences between survivals of the bacteria clustered into the most and less tolerant groups were found.

When analysing the behaviour of each bacteria to different freezing temperatures we found that *Lact. paracasei* and *Lact. mali* showed significant differences in survival between -20 and -80ºC (p= 0.01 and p=0.031, respectively) and between -80 and -196ºC (p= 0.008 and p=0.047), but not between 20 and -196ºC (P values>0.05). Survival rates of *Lactobacillus hilgardii, Lact. plantarum* and *Leuc. mesenteroides* are not significantly different at the temperatures tested. Survival rates of *Lact. brevis* and *O. oeni* were not significantly different neither between -20 and -80ºC nor between -20 and -196ºC (P values>0.05), but they were between -80 and -196ºC (p=0.022 and p=0.005, respectively). Finally, *Ped. parvulus* survival percentages show significant differences among -20 and -196ºC (p=0.015) but not between -20 and -80 ºC nor -80 and -196ºC.
When analysing the behaviour of bacteria grouping them by genus, Kruskal Wallis one way ANOVA test showed that there were significant differences in survival between genera. Namely, significant differences were recorded by the next genera couples: *Pediococcus* and *Lactobacillus* (p=0.026), *Pediococcus* and *Leuconostoc* (p=0.001) and *Pediococcus* and *Oenococcus* (p=0.023), but not in the rest of the possible couples (Supplementary Figure 1). From the analysis of the averages from survival rates at different freezing temperatures, it can be deduced that *Pediococcus* bacteria were the bacteria more tolerant to freeze-drying whatever freezing temperature used. The null

hypothesis that cell morphology did not influence survival rate must be retained as no significant differences from were found between survival rates of cocci and rod cells after statistical analysis (Supplementary Figure 2).

Discussion

Among factors determining the survival rate after freeze-drying, the type of microorganisms is one of the most important intrinsic factors, whereas freezing protocol is one of the most relevant extrinsic elements (Tsvetkov and Shishkova 1982; Santivarangkna *et al.* 2008). In this work, focused on the effect of freeze-drying on microorganism related to the winemaking process, we have observed important differences on survival of yeast and lactic acid bacteria: yeasts were more sensitive to this preservation method than bacteria, as already demonstrated Miyamoto-Shinohara *et al.* (2006). We have demonstrated that the initial freezing temperature is a crucial factor determining the performance of freeze-drying in the case of yeasts. However LABs were less affected by this variable. Dumont *et al.* (2004) reported similar findings when they tested the effect of different freezing rates on survival of the yeasts *S. cerevisiae* and *Candida utilis,* and the bacterium *Lact. plantarum*. They found that the highest viabilities of *S. cerevisiae* (>80%) were achieved at both, low (5 ºC min^{-1}) and at very high (30000 ºC min^{-1}) cooling rates, whereas at intermediate cooling rates, viabilities ranged between 40 and 65%. Viabilities recorded by us for this species were much lower, in the same range of that reported by Miyamoto-Shinohara *et al.* (2010), and these viabilities decreased as freezing temperatures lowered. Zhao and Zhang found that the malolactic species *O. oeni* and *Lact. brevis* showed higher viability after freeze-drying when they were frozen at -65 ºC instead of -20 ºC before sublimation (Zhao and Zhang 2005; Zhao and Zhang 2009). However, in our

case it was the contrary, although differences between the two temperatures were too small to be significant. Results from our *Lact. plantarum* strain showed slightly higher survival at -80 ºC than at the other two temperatures, but differences were not significant. What it became clear from the literature is that freeze-drying affected more negatively than freezing both yeast and bacteria. This is not surprising because they suffered not only the deleterious effects of freezing but also those derived from vacuum drying. This last process exposes cell envelopes to a hydrophobic environment which alters the membrane permeability in a more drastic way than freezing. Bravo-Ferrada *et al.* (2015) compared by flow cytometry the percentage of damaged membranes in freeze-dried and frozen cells of *Lact. plantarum*, and deduced that the highest percentage corresponded to lyophilized cells. Similar results were reported by Russell and Stewart (1981) from studies on survival of three brewing yeasts: *S. cerevisiae*, *Saccharomyces uvarum* and *Saccharomyces diastaticus*. They demonstrated that survival of these yeasts was higher when they were frozen than when were freeze-dried. Very low survival rates after lyophilization of *S. cerevisiae* cells (lower than 0.02%) have been reported by various researchers (Atkin *et al.* 1949; Miyamoto-Shinohara *et al.* 2010). By the contrary, *Lact. plantarum* is able to overcome lyophilization stress rendering higher percentages of viable cells (Tsvetkov and Brankova 1983; de Valdez *et al.* 1985; Dumont *et al.* 2004; García-Alegría *et al.* 2004). We have found values of 8-9% survival of *Lact. plantarum* at the three temperatures tested, lower of those reported by the previous cited authors; however, the survival rates are strain dependent characteristics as Bravo-Ferrada *et al.* (2015) have demonstrated.

We have found important differences in abilities to survive not only among yeasts and bacteria, but also between different strains belonging to each of these two groups. Several authors have found that even different strains of the same species may respond differently to the same preservation method (Prakash *et al.* 2013; Bravo-Ferrada *et al.* 2015). Taken

into account that factors as cooling rates, growth stage of the culture, type of lyoprotectant used, and composition of the suspension and re-hydration media were common for each yeast and bacterium, reasons explaining differences in survival must be related to distinguishing features existing between species, such as cell size and morphology, water content, membrane and cell wall composition, cell permeability, production of exopolysaccharides, or ability to metabolize different carbohydrates, as have been demonstrated by several authors (Dumont *et al.* 2004; C. Santivarangkna 2008; Miyamoto-Shinohara *et al.* 2008; Miyamoto-Shinohara *et al.* 2010; Prakash *et al.* 2013). Miyamoto-Shinohara *et al.* (2010) found a relationship between osmotolerance of yeasts and tolerance to freeze-drying. We have observed that the strains of species *P. membranifaciens*, *M. pulcherrima, T. delbrueckii, H. uvarum, P. kudriavzevii* and *W. anomalus*, all of them reported as osmotolerant (Wang *et al.* 2015), were more resistant to freeze-drying than the rest of yeasts. Although Wang *et al.* (2015) described *S. cerevisiae* as a species able to resist high sugar concentrations, Tofalo *et al.* (2009) described that osmotolerance is a strain dependent character in this species. We ignore if the low viability of the strain ENOLAB 2056 after freeze-drying is or not a consequence of its low osmotolerance because we have not tested this feature, but other authors have also found very low survival rates to freeze-drying of *S. cerevisiae* strains, especially when freezing is performed by dipping it in liquid nitrogen (-196ºC) (Wellman and Stewart 1973; Miyamoto-Shinohara *et al.* 2006). Hernández-López *et al.* (2003) tested the abilities of *T. delbrueckii* to survive and to leaven sweet and frozen sweet dough and found that that it was more tolerant to freezing and to overcome osmotic stress than *S. cerevisiae* strains. They concluded that survival differences between these two yeasts were related to the low invertase activity, the slow rate of trehalose mobilization, and with the ability to adapt rapidly to high osmotic pressures environments that exhibits *T. delbrueckii*.

As also pointed Miyamoto-Shinohara *et al.* (2010), we have observed that size of yeast could be one of the reasons that explain the differences in survival in the yeast group: the larger was the yeast, the lower survival showed; sizes of the yeasts tested in our work were obtained from Kurtzman and Fell (1998). So, *P. membranifaciens*, *S. bacillaris, M. pulcherrima,* and *T. delbrueckii* strains were more resistant to freeze-drying than the larger yeasts *H. uvarum*, *S. pombe* or *D. bruxellensis*. We did not notice a size effect on the LAB survival, possibly because differences in size among bacteria were very small. Data of bacterial sizes were obtained from Bergey's Manual of Systematic Bacteriology (Dicks and Holzapfel 2009; Hammes and Hertel 2009; Holzapfel *et al.* 2009a; Holzapfel *et al.* 2009b). Cell morphology did not explain differences although a higher number of species having coccus and bacillar shape should be tested to support our results.

As has been pointed by other authors, cell wall and membrane composition greatly affects survival after freeze-drying in both yeast and bacteria (Fernández Murga *et al.* 2001; Santivarangkna *et al.* 2008; Miyamoto-Shinohara *et al.* 2010). In general, yeast walls are composed of 85-90% polysaccharide and 10-15% protein. The polysaccharide component consists of a mixture of water-soluble mannan, alkali-soluble glucan, alkali-insoluble glucan and small amounts of chitin (Nguyen *et al.* 1998). Most of the protein is covalently linked to the mannan, which is more correctly described as mannoprotein. The proportions of these different components vary with the species and they have important influence on rheological properties of cell wall and membrane organization (Nguyen *et al.* 1998), which can influence its response to freezing and freeze-drying (Santivarangkna *et al.* 2008). Miyamoto-Shinohara *et al.* (2010) stated that the higher amount of glucan was present in the yeast walls, the higher survival rate after lyophilization was observed. However, this glucan can trap moisture, which decreased survival during storage. Moisture retention also occurs when yeasts are able to produce extracellular polysaccharides and has the same

effect that glucan (Miyamoto-Shinohara *et al.* 2010). Differences in peptidoglycan composition could explain differences in the response to freezing that we have found in freeze-dried LABs; thus, bacteria having meso-diamino-pimelic in their cell walls (*Lact. paracasei* and *Lact. mali*) tolerated significantly better freezing at -196ºC than at -80ºC. However, cells containing Lys-D-Asp (*Lact. brevis, Lact. hilgardii, Lact. plantarum* and *Ped. parvulus*) in their cell walls did not show significant differences in survival among the three freezing temperatures. Data from cell wall composition were obtained from Bergey's Manual of Systematic Bacteriology (Dicks and Holzapfel 2009; Hammes and Hertel 2009; Holzapfel *et al.* 2009a; Holzapfel *et al.* 2009b).

We have observed that survival rate of *P. kudriavzevii* (syn. *Issatchenkia orientalis*) is 100 and 300 times higher than *I. occidentalis* and *S. cerevisiae*. Similar differences between *S. cerevisiae* and *I. orientalis* were found by Miyamoto-Shinohara *et al.* (2010). It is generally accepted that freezing in liquid nitrogen (-196 ºC) was the method that resulted in the lowest viability of yeast strains compared to higher freezing temperatures (Wellman and Stewart 1973; Uzunova-Doneva and Donev 2000-2002; Abadias *et al.* 2001; Dumont *et al.* 2004). The reason why this occurs is because the freezing rate at this temperature (300 ºC min^{-1} from Uzunova-Doneva and Donev (2000-2002)) is too fast to let the internal water migrate outside the cell, and the water frozen inside the cell resulted in lethal damage (Abadias *et al.,* 2001). In the case of bacteria, in general, survival rates at -196ºC are a little higher than at lower temperatures. This differential fact related to the yeasts could be associated to the surface/volume (S/V) ratio, which determines thermal and water flow during freezing. Bacterial cells are smaller than yeast cells and they have an S/V ratio five times larger and, hence, water and heat will flow out faster from bacteria preventing in this way intracellular crystallization, which results in a higher viability. Cell wall and plasmatic

membrane differences between bacteria and yeast can also account for differences in survival (Dumont *et al.* 2004; Miyamoto-Shinohara *et al.* 2010).

From the results obtained in this work, we recommend that the freezing step during freeze-drying should be performed at -20ºC for yeast and *O. oeni* and *Ped. parvulus*, and at -196ºC for the rest of LABs, in order to obtain the best survival rates.

It has been recognized that survival of freeze-dried yeast is lower than survivals obtained from other preservation methods as L-drying, drying or freezing (Atkin *et al.* 1949; Miyamoto-Shinohara *et al.* 2010; Prakash *et al.* 2013). Although liquid fresh or frozen yeast starters provide higher viable cell population, the short time frame of the fresh cultures or the difficulties for distribution and storage of the frozen yeast justifies that, the majority of yeast starter cultures used for food fermentations are sold as active dried yeasts (Aguilera and Karel 1997; Krieger-Weber 2009). However, LAB dried starters are practically inexistent in the market, possibly due to the low tolerance of these bacteria to drying. Although there are not many works that compare the effects of drying and freeze-drying, Kim and Bhowmik (1990) found that spray-dried yogurt powder showed lower survival for *Staphylococcus thermophilus* than freeze-dried powder. This fact, besides a higher storage stability, and an easy handling during storage, distribution and application, makes freeze-drying be preferable to freezing to produce commercial starters, instead of frozen cultures have higher percentage of viable cells and that cells need shorter time for activation (Buckenhüskes 1993).

Data obtained in these experiments are important from the practical point of view, as optimal temperatures to perform freezing previous to freeze-drying can be deduced. Data obtained in these experiments are important from the practical point of view. Setting

freezing at -20ºC ensures higher values of yeast viability, reaching a maximum of 5 log units related to -196ºC, whereas this last temperature ensures up to 5 times more survival in LABs compared to -20ºC. Optimization of freeze-drying conditions ensures a greater security in preserving microorganisms of industrial importance and helps to define guidelines for improving the performance of commercially produced starter cultures' preparations.

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Conflict of interest

No conflict of interest declared

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Figure legends:

Figure 1.- Boxplot resulting from Kruskal-Wallis ANOVA one way test considering survival rates as independent variable and type of microorganism as factor. Asterisk show extreme data.

Figure 2.- Boxplot resulting from Kruskal-Wallis ANOVA one way test considering yeast survival as independent variable and freezing temperature as factor. Asterisk and circle marks extreme data.

Figure 3.- Boxplot resulting from Kruskal-Wallis ANOVA one way test considering LAB survival as independent variable and freezing temperature as factor.

Figure 4.- Boxplot resulting from Kruskal-Wallis ANOVA one way test considering yeast survival as independent variable and yeast strain as factor.

Figure 5.- Boxplot resulting from Kruskal-Wallis ANOVA one way test considering survival rate as independent variable and bacterial strain as factor. Asterisk and circle label extreme data.

Supplementary Figure 1: Boxplot resulting from Kruskal-Wallis ANOVA one-way test considering survival rate as independent variable and genus as factor.

Supplementary Figure 2: Boxplot resulting from Kruskal-Wallis ANOVA one-way test considering survival rate as independent variable and cell morphology as factor.

Table 1: Yeast and LAB strains used in this study

Species	**Strain number**
Dekkera bruxellensis	CECT 1451^{T}
Hanseniaspora uvarum	CECT 1444^{T}
Issatchenkia occidentalis	CECT 11204^{T}
Metschnikowia pulcherrima	CECT 11202^{T}
Pichia kudriavzevii	CECT 10688^{T}
Pichia membranifaciens	CECT 11982^{T}
Saccharomyces cerevisiae	ENOLAB 5021
Schizosaccharomyces pombe	CECT 10685^{T}
Starmerella bacillaris	CECT 11046
Torulaspora delbrueckii	CECT 1015
Wickerhamomyces anomalus	CECT 1114^{T}
Lactobacillus brevis	ENOLAB 3810
Lactobacillus hilgardii	ENOLAB 3808
Lactobacillus mali	ENOLAB 3812
Lactobacillus paracasei	ENOLAB 3806

Lactobacillus plantarum	CECT 748^{T}
Leuconostoc mesenteroides	ENOLAB 4605
Oenococcus oeni	ENOLAB 4168
Pediococcus parvulus	ENOLAB 3908

Table 2: Survival rates of yeasts lyophilized at different temperatures. [a]: Average of survival rates obtained at -20, -80 and -196ºC for every yeast. [b]: Average of survival rates obtained for all yeasts at -20ºC. [c]: Average of survival rates obtained for all yeasts at -80ºC. [d]: Average of survival rates obtained for all yeasts at -196ºC. [b]: Average of survival rates obtained for all yeasts at -20, 80 and -196ºC.

Yeast species	**Survival percentage (± SD)**			
	-20 ºC	**-80 ºC**	**-196 ºC**	**Average[a]**
Pichia membranifaciens	3.8×10^{1} ($\pm 8.0 \times 10^{0}$)	2.7×10^{1} ($\pm 2.1 \times 10^{0}$)	1.0×10^{0} ($\pm 0.2 \times 10^{0}$)	2.2×10^{1} ($\pm 3.4 \times 10^{0}$)
Starmerella bacillaris	3.1×10^{1} ($\pm 1.0 \times 10^{0}$)	2.5×10^{1} ($\pm 3.7 \times 10^{0}$)	1.7×10^{-3} ($\pm 3.4 \times 10^{-4}$)	1.8×10^{1} ($\pm 1.6 \times 10^{0}$)
Metschnikowia pulcherrima	2.5×10^{1} ($\pm 3.9 \times 10^{0}$)	2.1×10^{1} ($\pm 2.9 \times 10^{0}$)	2.9×10^{0} ($\pm 1.1 \times 10^{0}$)	1.6×10^{1} ($\pm 2.6 \times 10^{0}$)
Torulaspora delbrueckii	6.8×10^{0} ($\pm 0.8 \times 10^{0}$)	2.5×10^{0} ($\pm 0.7 \times 10^{0}$)	2.8×10^{-2} ($\pm 4.4 \times 10^{-3}$)	3.1×10^{0} ($\pm 0.5 \times 10^{0}$)
Hanseniaspora uvarum	2.8×10^{0} ($\pm 3.5 \times 10^{-2}$)	1.9×10^{0} ($\pm 0.1 \times 10^{0}$)	3.0×10^{-2} ($\pm 2.8 \times 10^{-3}$)	1.6×10^{0} ($\pm 0.4 \times 10^{-1}$)
Pichia kudriavzevii	9.0×10^{-1} ($\pm 4.7 \times 10^{-2}$)	1.1×10^{0} ($\pm 0.3 \times 10^{0}$)	0.2×10^{0} ($\pm 8.8 \times 10^{-2}$)	7.0×10^{-1} ($\pm 0.1 \times 10^{0}$)

Wickerhamomyces anomalus	2.0×10^{-1} ($\pm 6.5 \times 10^{-2}$)	8.8×10^{-2} ($\pm 7.5 \times 10^{-3}$)	4.4×10^{-3} ($\pm 1.4 \times 10^{-3}$)	9.0×10^{-1} ($\pm 0.2 \times 10^{-1}$)
Schizosaccharomyces pombe	6.3×10^{-2} ($\pm 3.6 \times 10^{-2}$)	5.1×10^{-4} ($\pm 1.8 \times 10^{-4}$)	1.1×10^{-3} ($\pm 2.0 \times 10^{-4}$)	6.3×10^{-4} ($\pm 1.2 \times 10^{-2}$)
Issatchenkia occidentalis	8.8×10^{-3} ($\pm 6.5 \times 10^{-4}$)	8.3×10^{-4} ($\pm 2.0 \times 10^{-4}$)	5.9×10^{-4} ($\pm 2.3 \times 10^{-4}$)	3.4×10^{-3} ($\pm 3.6 \times 10^{-4}$)
Saccharomyces cerevisiae	3.1×10^{-3} ($\pm 4.9 \times 10^{-4}$)	2.0×10^{-3} ($\pm 1.8 \times 10^{-4}$)	3.1×10^{-4} ($\pm 5.4 \times 10^{-6}$)	1.8×10^{-3} ($\pm 2.3 \times 10^{-4}$)
Dekkera bruxellensis	1.8×10^{-4} ($\pm 6.5 \times 10^{-5}$)	1.5×10^{-3} ($\pm 1.0 \times 10^{-4}$)	3.4×10^{-4} ($\pm 1.2 \times 10^{-4}$)	6.6×10^{-4} ($\pm 9.6 \times 10^{-5}$)
Average	9.45×10^{1} ($\pm 1.3 \times 10^{0}$) [b]	7.06×10^{1} ($\pm 0.9 \times 10^{0}$) [c]	0.37×10^{1} ($\pm 1.3 \times 10^{0}$) [d]	5.6×10^{0} ($\pm 1.2 \times 10^{0}$) [e]

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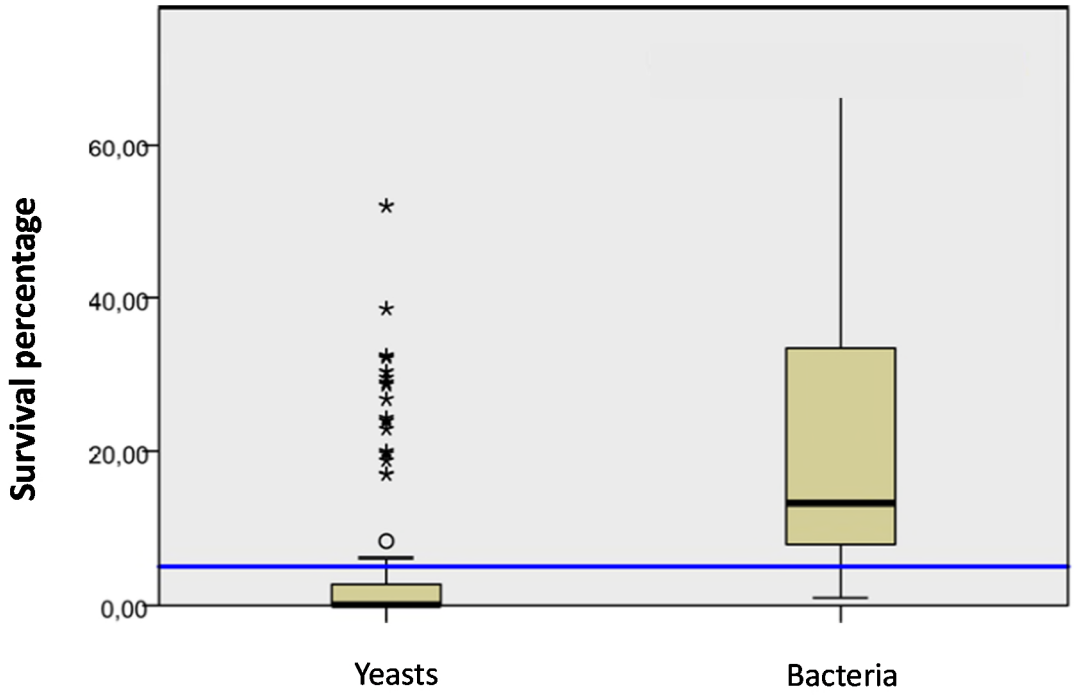
Table 3: Survival rates obtained after lyophilization of LABs at different temperatures. [a]: Average of survival rates obtained at -20, -80 and -196ºC for every LAB. [b]: Average of survival rates for all LABs obtained at -20ºC. [c]: Average of survival rates obtained for all LABs at -80ºC. [d]: Average of survival rates obtained for all LABs at -196ºC. [b]: Average of survival rates obtained for all LABs at -20, 80 and -196ºC.

LAB strain	**Survival percentage (± SD)**			
	-20 °C	**-80 °C**	**-196 °C**	**Average[a]**
Lactobacillus paracasei	4.8×10^1 ($\pm 2.5 \times 10^0$)	3.4×10^1 ($\pm 1.8 \times 10^0$)	5.2×10^1 ($\pm 5.8 \times 10^0$)	4.5×10^1 ($\pm 8.6 \times 10^0$)
Pediococcus parvulus	4.2×10^1 ($\pm 2.9 \times 10^0$)	4.1×10^1 ($\pm 1.3 \times 10^1$)	3.1×10^1 ($\pm 3.9 \times 10^0$)	3.8×10^1 ($\pm 9.0 \times 10^0$)
Lactobacillus mali	2.6×10^1 ($\pm 2.5 \times 10^0$)	1.9×10^1 ($\pm 1.5 \times 10^1$)	5.1×10^1 ($\pm 1.4 \times 10^1$)	3.2×10^1 ($\pm 1.8 \times 10^1$)
Oenococcus oeni	2.1×10^1 ($\pm 4.1 \times 10^0$)	1.2×10^1 ($\pm 4.0 \times 10^0$)	7.3×10^0 ($\pm 0.3 \times 10^0$)	1.3×10^1 ($\pm 3.5 \times 10^0$)
Lactobacillus hilgardii	1.3×10^1 ($\pm 1.5 \times 10^0$)	1.4×10^1 ($\pm 2.1 \times 10^0$)	1.7×10^1 ($\pm 4.8 \times 10^0$)	1.5×10^1 ($\pm 3.1 \times 10^0$)
Leuconostoc mesenteroides	8.3×10^0 ($\pm 1.0 \times 10^0$)	8.9×10^0 ($\pm 1.2 \times 10^0$)	8.9×10^0 ($\pm 1.4 \times 10^0$)	1.3×10^1 ($\pm 6.3 \times 10^0$)
Lactobacillus plantarum	8.2×10^0 ($\pm 0.8 \times 10^0$)	9.5×10^0 ($\pm 2.6 \times 10^{-0}$)	8.0×10^0 ($\pm 1.8 \times 10^0$)	8.6×10^0 ($\pm 6.8 \times 10^0$)

Lactobacillus brevis	2.3×10^0 ($\pm$ 0.9×10^0)	1.4×10^0 ($\pm$ 0.2×10^0)	1.2×10^1 ($\pm$ 6.3×10^0)	5.1×10^0 ($\pm$ 5.9×10^0)
Average[b]	2.1×10^1 ($\pm$ 2.0×10^0)	1.8×10^1 ($\pm$ 5.0×10^0)	2.3×10^1 ($\pm$ 4.7×10^0)	2.1×10^1($\pm$ 7.7×10^0)

11

12



13

14 Figure 1

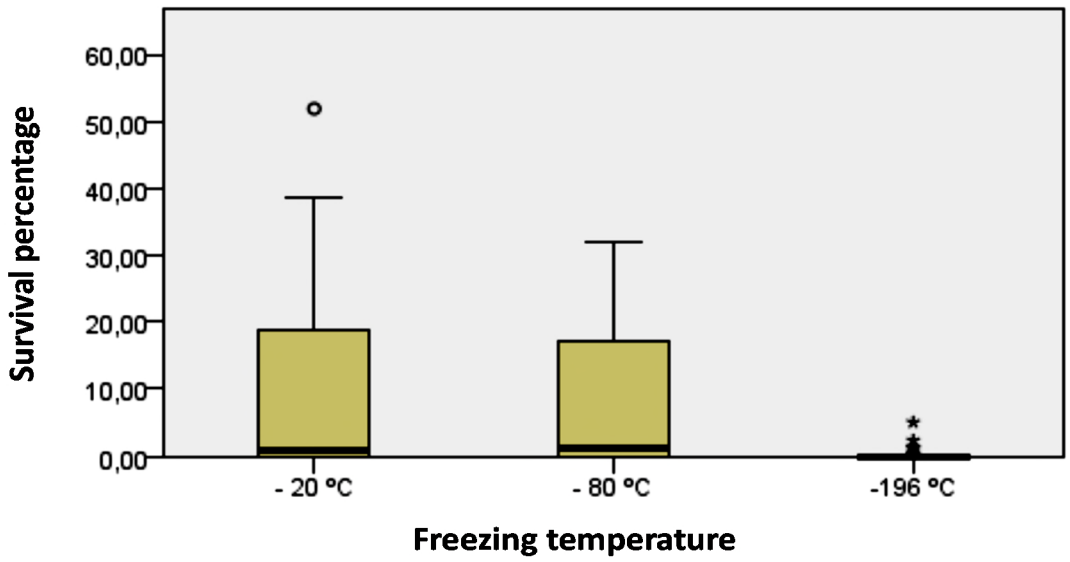
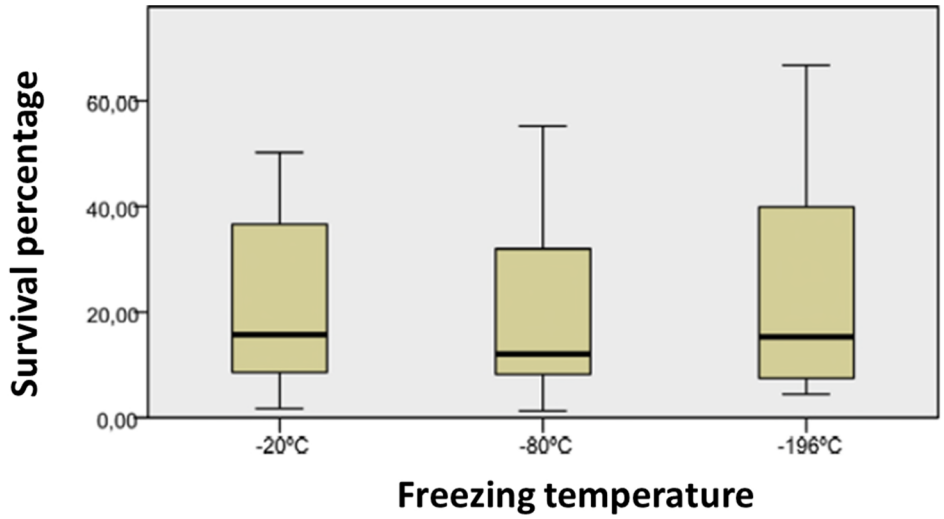

Survival percentage
60,00
50,00
40,00
30,00
20,00
10,00
0,00
- 20 °C
- 80 °C
-196 °C
Freezing temperature

Figure 2



18

19 Figure 3

20

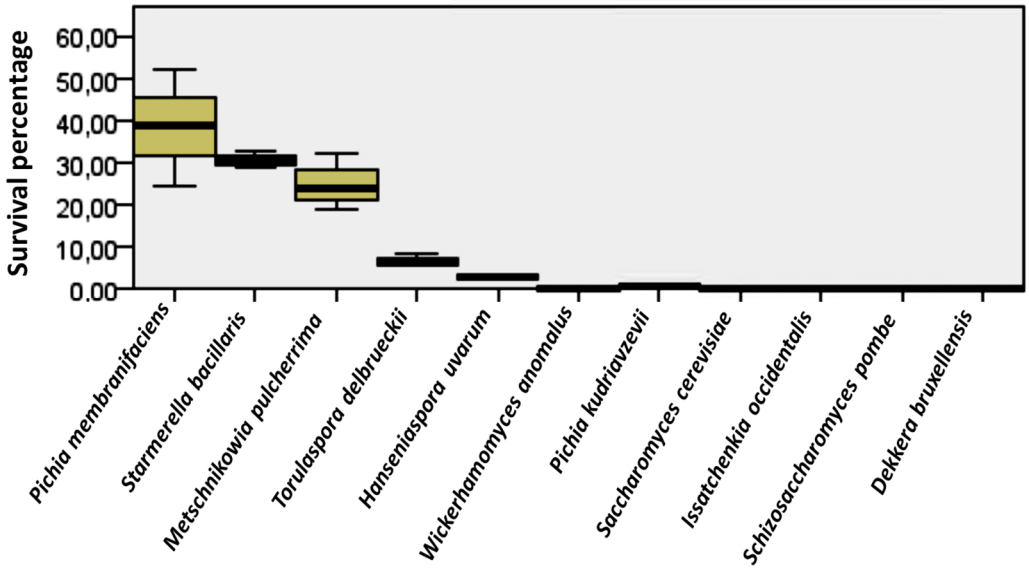
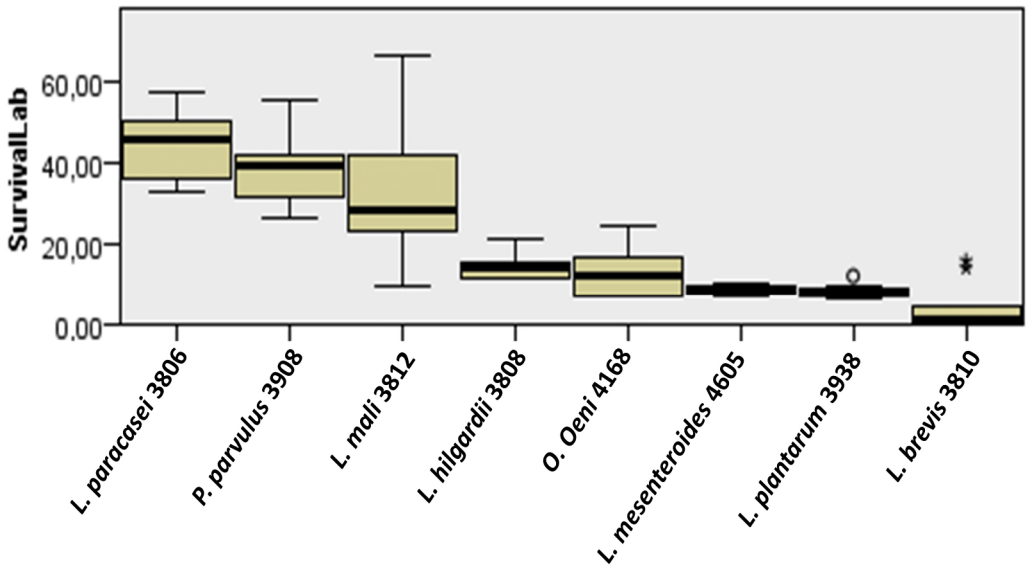

Survival percentage
60,00
50,00
40,00
30,00
20,00
10,00
0.00
Pichia membranifaciens
Starmerella bacillaris
Metschnikowia pulcherrima
Torulaspora delbrueckii
Hanseniaspora uvarum
Wickerhamomyces anomalus
Pichia kudriavzevii
Saccharomyces cerevisiae
Issatchenkia occidentalis
Schizosaccharomyces pombe
Dekkera bruxellensis

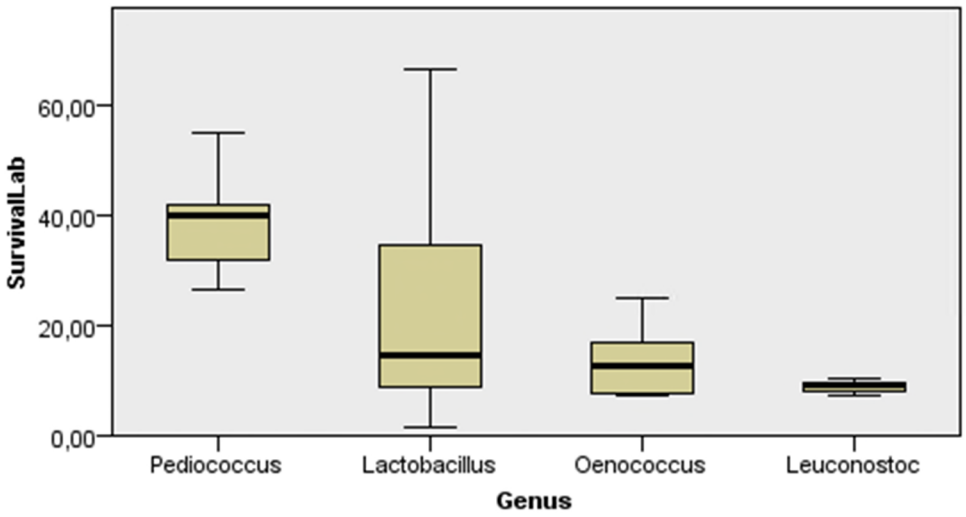
Figure 4



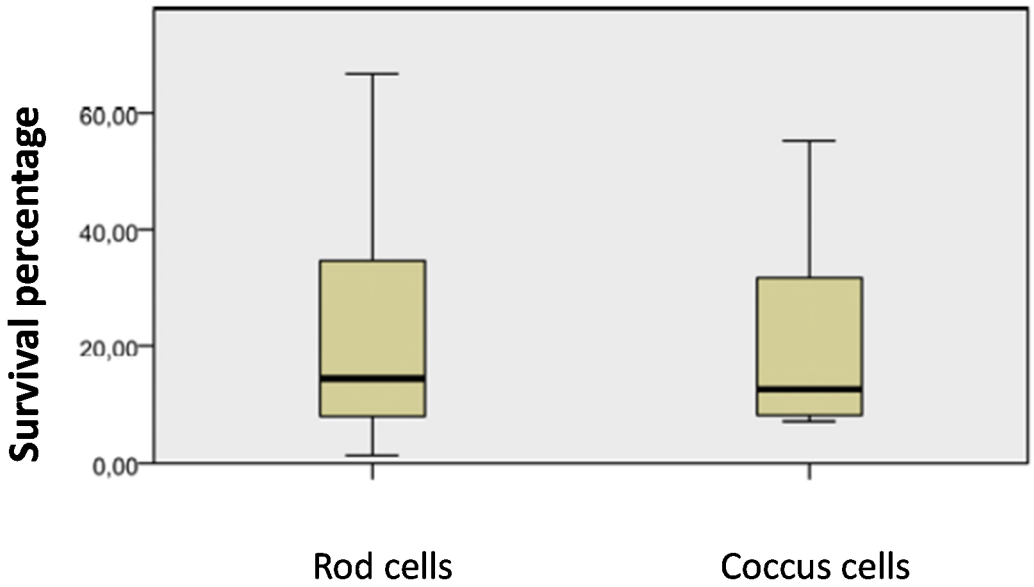
25

26 Figure 5

27



Supplementary Figure 1



Supplementary Figure 2