Influence of freezing temperatures prior to freeze-drying on viability of yeasts and lactic acid bacteria isolated from wine

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Abstract

Aims: To determine the effect of three different freezing temperatures on post-freeze-drying survival rates of wine yeasts and lactic acid bacteria (LAB). To know if a similar freeze-drying protocol can be used for both micro-organisms.

Methods and Results: Cells from liquid culture media were recovered and concentrated in appropriate lyoprotectants. Aliquots of each strain were frozen at −20, −80 and −196°C before vacuum drying. Viable cell counts were done before freezing and after freeze-drying. Survival rates were calculated. Freezing temperatures differently affected yeast and bacteria survival. The highest survival rates were obtained at −20 and −80°C for yeasts, but at −196°C for LAB. Major differences in survival rates were recorded among freeze-dried yeasts, but were less drastic for LAB. Yeasts Pichia membranifaciens, Starmerella bacillaris and Metschnikowia pulcherrima, and LAB Lactobacillus paracasei, Pediococcus parvulus and Lactobacillus mal, were the most tolerant species to freeze-drying, regardless of freezing temperature.

Conclusions: Yeast and LAB survival rates differed for each tested freezing temperature. For yeasts, −20°C ensured the highest post-freeze-drying viability and −196°C for LAB.

Significance and Impact of the Study: Freezing temperature to freeze-dry cells is a crucial factor for ensuring good wine yeast and LAB survival. These results are important for appropriately preserving micro-organisms and for improving starter production processes.

Introduction

Yeast and lactic acid bacteria (LAB) are involved in numerous traditional European, African and Asian food fermentations that result in either basic products, like bread, sausages, dairy products, sauerkraut, beverages or exotic products like gari, idli, ogi, etc. (Caplice and Fitzgerald 1999; Holzapfel 2002). Preserving the micro-organisms responsible for these fermentations is of much interest at both domestic and industrial levels. The careful preservation of micro-organisms is imperative for future research, teaching and industrial applications (Prakash et al. 2013). Freeze-drying is the preferred long-term preservation method used by microbial resource centres, and by industries that produce bacterial starters (Hosier et al. 1999; Krieger 2009; Prakash et al. 2013), given its easy transportation and use, low-cost maintenance and high cell survival rates over long periods (Miyamoto-Shinohara et al. 2006; Morgan et al. 2006; Prakash et al. 2013). Freeze-drying is a preservation form based on a cold-drying process that 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 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, lyoprotectant type, freezing, sublimation and thawing temperatures, degree of dehydration achieved, reconstitution medium, and time and storage, and rehydration conditions, have been described as strongly influencing the survival of micro-organisms (Donev et al. 1995; Dumont et al. 2004; Zhao and Zhang 2005; Nakamura et al. 2009; Peiren et al. 2015). Biological factors, such as micro-organism type, initial cell concentration, age of cells and the presence of compatible solutes in cytoplasm, can affect freeze-dried cell survival (Donev et al. 1995; Miyamoto-Shinohara et al. 2000). Freeze-drying always implies a drop in viable cells (Tymczyszyn et al. 2007) since it strongly affects 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 an increased internal solute concentration and macromolecule denaturation induced by water removal (Pehkonen et al. 2008). Although freezing itself has no lethal effect for cells, it can induce physical stress that can injure a part of these cells, and thus reduce 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, which thus causes extracellular water removal and an increased solute concentration which, in turn, leads to osmotic imbalance. Conversely, if freezing is too fast, cells cannot lose water fast enough to maintain the balance, and intracellular ice crystals can appear and can thus have damaging or even lethal effects (Seki et al. 2009).

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

The aim of this work was to evaluate the influence of different freezing temperatures, prior to vacuum drying, on the viability of wine-isolated micro-organisms preserved by freeze-drying. Nowadays, many winemakers prefer to substitute commercial yeast and LAB cultures for the micro-organisms isolated from their wineries (Berbegal et al. 2017). Using micro-organisms that are typical of the winery allows the production of more unique wines than those obtained with commercial cultures. Among yeasts, the interest lies in not only Saccharomyces cerevisiae but also in non-Saccharomyces strains, as these latter kind of yeasts can improve the organoleptic properties of wines. In addition to yeast, LAB are used to properly manage the acidity of wines by not only de-acidification but also by acidification (Lucio et al. 2016). Although Oenococcus oeni is the preferred bacterium to perform de-acidification by conducting malolactic fermentation, other wine LAB species can also perform this reaction (Lactobacillus plantarum, Lactobacillus brevis, Pediococcus parvulus, etc.) (Berbegal 2014). When acidification instead of de-acidification is intended, L. plantarum is one of the most promising bacteria to accomplish this task (Lucio et al. 2016).

The use of noncommercial cultures in winemaking requires a previous selection process (Berbegal et al. 2017) and proper preservation methods so that the selected cultures properties remain unaltered. Various research groups that work in wine microbiology have important culture collections that need to be properly preserved for future research and industrial use. Finally, although the companies that produce wine yeasts and LAB starters have standard protocols to produce their products, they always welcome new information about optimizing preservation methods. The aim of this work was to contribute to define the best conditions to perform freeze-drying of important wine micro-organisms, which is of paramount interest to research and private sectors.

Materials and methods

Micro-organisms

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

Yeast biomass production

Yeast strains were grown in glucose peptone yeast (GPY) extract medium. Medium composition per litre was as follows: 20 g glucose (Panreac, Barcelona, Spain), 10 g acid casein peptone H (Pronadisa, Madrid, Spain), yeast extract 5 g (Pronadisa) and agar 20 g (Pronadisa). The pH of the medium was adjusted to 5.5. Next, 100-ml cultures were grown at 28°C until the mid-stationary phase under shaking conditions at 160 rpm in a HT Infor AG rotatory shaker. Yeast cells were harvested by aseptic centrifugation at 6000 rev min⁻¹ (6842 g) for 15 min at 4°C in a Multifuge 1 S-R centrifuge (Heraeus, Hanau, Germany). The pellet was washed with 100 ml of 0.9% NaCl (Panreac) solution, centrifuged again under the same
conditions and concentrated in 3 ml of a lyoprotectant solution that consisted of 2 ml of skimmed milk powder (Oxoid, Basingstoke, UK) and 1 ml of 15% glucose (Panreac) solutions.

**Bacterial biomass production**

The *Lactobacillus*, *Pediococcus* and *Leuconostoc* species were grown in the de Man, Rogosa and Sharpe (MRS) medium (Scharlab) supplemented with L-Cysteine (0.5 g l⁻¹) (Sigma, Madrid, Spain). *Oenococcus oeni* was grown in *Leuconostoc oenos* medium (MLO) at pH 4.8 (Zúñiga et al. 1993). The 100-ml cultures were statically incubated at 28°C until the mid-stationary phase in a Schott flask with a small air head space. Bacterial cells were harvested by aseptic centrifugation at 6000 rev min⁻¹ (6842 g) for 15 min at 4°C in a Multifuge 1 S-R centrifuge (Heraeus), washed with the same volume of glutamic acid (0.067 mol l⁻¹) and centrifuged again 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**

Three hundred-microlitre volumes of cell suspensions were distributed into 0.5-mm-diameter sterile glass tubes. After 10 min at room temperature, they were subjected to different freezing treatments: −20°C for 1 h, −80°C for 1 h and −196°C (by immersion in liquid N₂) for 1 min. Immediately after treatments, cells were freeze-dried in a Virtis lyophiliser that operated at a 15-9 mTorr vacuum pressure for 18 h. After freeze-drying, glass tubes were sealed at a vacuum pressure of 1.7 mTorr, and then stored at 4°C for 1 week. After this storage period, cells were rehydrated and homogenized with 300 µl of the appropriate culture media: GPY for yeasts, MRS for the *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, according to micro-organism type. Concentrated cell suspensions, taken immediately before freezing and after rehydration, were serially diluted and spread on plates. Plates were incubated at 28°C for 3–4 days. Then the number of colony-forming units per millilitre (CFU per ml) was determined. The survival percentage was calculated as the percentage of viable cells after freeze-drying in relation to viable cells before freeze-drying. The survival assays were carried out in triplicate.

**Statistical analysis**

Statistical analyses were performed with v22.0.0.1 of the Statistical Package for Social Sciences (SPSS) programme (SPSS Inc., Chicago, IL, USA). Generally speaking, the independent variable (survival rates) and the dependent variables’ (micro-organism type, strain, freezing treatment, bacterial cell shape and bacterial genus) distribution was assessed by a Kruskal–Wallis one-way ANOVA, a nonparametric test. Pairwise comparisons were calculated to investigate the relationship between different species (yeast or bacteria). The confidence interval for a difference in medians was set at 95% (*P* ≤ 0.05) for all comparisons.

**Results**

**Effect of freezing temperatures on yeast survival after freeze-drying**

As observed in Table 2, the survival rates after freeze-drying varied widely among yeasts. The extreme values corresponded to *Pichia membranifaciens* frozen at −20°C (38%), and to *Dekkera bruxellensis* frozen at the same temperature (0.00018%). The highest survival rates were generally obtained at −20°C and the lowest ones at −196°C. At −80°C, the most and the least resistant yeasts were *P. membranifaciens* and *Schizosaccharomyces pombe*, whereas *Metschnikovia pulcherrima* and *S. cerevisiae* were the most and the least resistant at −196°C (both

| Species                          | Strain number   |
|---------------------------------|-----------------|
| Dekkera bruxellensis            | CECT 1451T      |
| Hanseniaspora uvarum            | CECT 1444T      |
| Issatchenkia occidentalis       | CECT 11204T     |
| Metschnikovia pulcherrima       | CECT 11202T     |
| Pichia kudriazewii              | CECT 10688T     |
| Pichia membranifaciens          | CECT 11982T     |
| Saccharomyces cerevisiae         | ENOLAB 5021     |
| Schizosaccharomyces pombe       | CECT 10685T     |
| Starmerella bacillaris          | CECT 11046      |
| Torulaspora delbrueckii         | CECT 1015       |
| Wickerhamomyces anomalous       | CECT 1114T      |
| Lactobacillus brevis            | ENOLAB 3810     |
| Lactobacillus hilgardii         | ENOLAB 3808     |
| Lactobacillus mali              | ENOLAB 3812     |
| Lactobacillus paracasei         | ENOLAB 3806     |
| Lactobacillus plantarum         | CECT 748T       |
| Leuconostoc mesenteroides       | ENOLAB 4605     |
| Oenococcus oeni                 | ENOLAB 4168     |
| Pediococcus pentosus            | ENOLAB 3908     |

1Type strain.
Table 2 Survival percentages of the yeasts freeze-dried at different temperatures

| Yeast species                  | Counts before freezing* (±SD) | −20°C  | −80°C  | −196°C |
|--------------------------------|-------------------------------|--------|--------|--------|
| *Pichia membranifaciens*       | 3.5 × 10⁶ (±1.2 × 10⁵)        | 3.8 × 10⁵ (±8.0 × 10⁵) | 2.7 × 10⁵ (±2.1 × 10⁵) | 1.0 × 10⁵ (±0.2 × 10⁵) |
| *Starmerella bacillaris*       | 2.0 × 10¹⁰ (±1.1 × 10¹⁰)      | 3.1 × 10⁴ (±1.0 × 10⁵) | 2.5 × 10⁴ (±3.7 × 10⁵) | 1.7 × 10⁻³ (±3.4 × 10⁻⁴) |
| *Metschnikowia pulcherrima*    | 1.1 × 10¹⁰ (±1.3 × 10¹⁰)      | 2.5 × 10⁴ (±3.9 × 10⁴) | 2.1 × 10⁴ (±2.9 × 10⁴) | 2.9 × 10⁰ (±1.1 × 10⁰) |
| *Torulaspora delbrueckii*      | 4.7 × 10¹⁰ (±2.6 × 10⁹)       | 6.8 × 10⁵ (±0.8 × 10⁶) | 2.5 × 10⁵ (±0.7 × 10⁵) | 2.8 × 10⁻² (±4.4 × 10⁻³) |
| *Hanseniaspora uvarum*         | 8.4 × 10¹⁰ (±1.3 × 10¹⁰)      | 2.8 × 10⁵ (±3.5 × 10⁵) | 1.9 × 10⁵ (±0.1 × 10⁵) | 3.0 × 10⁻² (±2.8 × 10⁻³) |
| *Pichia kudriavzevii*          | 1.9 × 10⁷ (±2.4 × 10⁶)        | 9.0 × 10⁻¹ (±4.7 × 10⁻⁴) | 1.1 × 10⁰ (±0.3 × 10⁰) | 2.0 × 10⁻¹ (±8.8 × 10⁻³) |
| *Wickerhamomyces anomalus*     | 6.9 × 10¹⁰ (±9.4 × 10⁹)       | 2.0 × 10⁻¹ (±6.5 × 10⁻¹) | 8.8 × 10⁻² (±7.5 × 10⁻³) | 4.4 × 10⁻³ (±1.4 × 10⁻³) |
| *Schizosaccharomyces pombe*    | 4.1 × 10⁸ (±5.2 × 10⁷)        | 6.3 × 10⁻² (±3.6 × 10⁻²) | 5.1 × 10⁻⁴ (±1.8 × 10⁻⁴) | 1.1 × 10⁻³ (±2.0 × 10⁻⁴) |
| *Issatchenkia occidentalis*    | 1.7 × 10¹¹ (±3.1 × 10¹⁰)      | 8.8 × 10⁻³ (±6.5 × 10⁻⁴) | 8.3 × 10⁻⁴ (±2.0 × 10⁻⁴) | 5.9 × 10⁻⁴ (±2.3 × 10⁻⁴) |
| *Saccharomyces cerevisiae*     | 1.3 × 10¹⁰ (±2.4 × 10⁹)       | 3.1 × 10⁻³ (±4.9 × 10⁻⁴) | 2.0 × 10⁻³ (±1.8 × 10⁻⁴) | 3.1 × 10⁻⁴ (±5.4 × 10⁻⁶) |
| *Dekkera bruxellensis*         | 6.3 × 10⁹ (±1.9 × 10⁸)        | 1.8 × 10⁻⁴ (±6.5 × 10⁻⁵) | 1.5 × 10⁻³ (±1.0 × 10⁻⁴) | 3.4 × 10⁻⁴ (±1.2 × 10⁻⁴) |
| Average                        | 5.6 × 10¹⁰ (±6.0 × 10⁹)       | 9.5 × 10⁰ (±1.3 × 10⁰)† | 7.1 × 10⁰ (±0.9 × 10⁰)‡ | 3.8 × 10⁻¹ (±1.3 × 10⁻¹) § |

*Counts of the concentrated cell suspension.
†Average of the survival rates obtained for all the yeasts at −20°C.
‡Average of the survival rates obtained for all the yeasts at −80°C.
§Average of the survival rates obtained for all the yeasts at −196°C.

respectively). Figure 1 contains boxplots that summarize the behaviours of the 11 yeasts against freezing temperatures. By taking into account the survival rate of yeast at −20, −80 and −196°C, and freezing temperature as a factor, different scenarios were found after applying the Kruskal–Wallis one-way ANOVA. Significant differences in the medians of the survival rates between −20 and −196°C were found for yeasts *P. membranifaciens* (*P* = 0.05), *Torulaspora delbrueckii* (*P* = 0.022), *Hanseniaspora uvarum* (*P* = 0.022), *Wickerhamomyces anomalus* (*P* = 0.05) and *S. cerevisiae* (*P* = 0.022) (Fig. 1: A, D, E, F, H), but not between −20 and −80°C, nor between −80 and −196°C. The *S. pombe* medians of the survival rates at −20 and −80°C significantly differed (*P* = 0.034), but not between −20 and −196°C, nor between −80 and −196°C (Fig. 1: J). No significant differences among the medians of the survivals at the three different temperatures were found for the other yeasts (Fig. 1: B, C, G, I and K).

As deduced from Table 2 and Fig. 1, the highest yeast survival rates were generally found at −20°C (38–0.0018%), with the lowest ones at −196°C (2.9–0.00031%). We found that the survival rates at −20°C were between 90 and 99.99% higher than at −196°C. In the vast majority of cases, yeast survival decreased as freezing temperature lowered, except for *Pichia kudriavzevii* (1G) and *D. bruxellensis* (1K), for which survival at −80°C was higher than at −20°C. The *P. kudriavzevii* survival rate at −20°C was 97.8% higher than at −196°C, whereas the *D. bruxellensis* survival rate at −20°C was slightly lower than at −196°C. These results revealed that wine yeasts behaved quite homogeneously vs freezing temperatures, with −196°C being the most harmful temperature for them and −20°C the least harmful one.

The boxplots that resulted from the Kruskal–Wallis analysis of the survival rates of yeasts at −20, −80 and −196°C indicate major differences between the medians of the survival rates of the different yeasts (Fig. 2). The Kruskal–Wallis one-way ANOVA analysis found significant differences in the medians of the survival rates of yeasts at −20°C (*P* = 0.001), −80°C (*P* = 0.001) and −196°C (*P* = 0.001). This means that the medians of the yeast survivals at each temperature were not equal, and that differences can be attributed to yeast type. Despite the evident differences in the medians of the survival rates displayed in Fig. 2, the analysis by yeast pair-wise comparisons showed that only the medians of the couples *D. bruxellensis*–*Starmerella bacillaris* (*P* = 0.029) and *D. bruxellensis*–*P. membranifaciens* (*P* = 0.016) significantly
differed at −20°C. At −80°C, significant differences in the medians were found for two couples: *S. pombe*–*S. bacillaris* \((P = 0.040)\) and *S. pombe*–*P. membranifaciens* \((P = 0.025)\). Finally, at −196°C, significant differences among the medians of the survival rates were found in couples *D. bruxellensis*–*M. pulcherrima* \((P = 0.018)\) and *S. cerevisiae*–*M. pulcherrima* \((P = 0.021)\). The most resistant yeasts to freeze-drying were the same, no matter what freezing temperature was used: *P. membranifaciens*, *S. bacillaris* and *M. pulcherrima*. The least resistant ones were *W. anomalus*, *S. cerevisiae*, *Issatchenkia occidentalis*, *S. pombe* and *D. bruxellensis* (Fig. 2). When considering the medians of the survival rates of the wine yeasts *vs* temperatures all together (Fig. 3), the Kruskal–Wallis one-way ANOVA analysis showed that the medians of the survival rates of yeasts were not equal and that differences were due to the freezing temperature effect \((P = 0.001)\). The analysis by a couple of temperatures showed significant differences in the medians of the survival rates that corresponded to −20 and −196°C \((P = 0.001)\), and between those that corresponded to −80 and −196°C \((P = 0.014)\), but not between those obtained at −20 and −80°C \((P = 1.000)\).

In short, yeast type and freezing temperature influenced the survival rates obtained after freeze-drying.

**Effect of freezing temperatures on LAB survival after freeze-drying**

The survival rates of the freeze-dried wine LAB are shown in Table 3. The LAB survival rates ranged between 52 and 1.4% (Table 3). The highest value corresponded to the *Lactobacillus paracasei* frozen at −196°C, and the lowest one went to *L. brevis* frozen at −80°C. In general, the survival rates at −196°C were higher than at the other temperatures, but only slightly. In fact, the survival percentages at −20 and −80°C were 91 and 78%, (averages), respectively, of those observed at −196°C. The most sensitive species to freezing were
The most resistant bacteria were L. paracasei at both −20 and −196°C, and P. parvulus at −80°C respectively. Figure 4 displays eight boxplots, which describe the behaviours of the different LAB vs the three tested freezing temperatures. The L. brevis (4H), Lactobacillus mali (4C), Lactobacillus hilgardii (4D) and L. paracasei (4A) survival rates were higher at −196°C than at the other two temperatures. Freezing at −80°C ensured the highest survival for L. plantarum (4G), whereas −20°C was the best treatment temperature for O. oeni (4E). The survival rates of Leuconostoc mesenteroides (4F) at −20 and −80°C were similar and slightly higher than those achieved at −196°C. The survival rates of P. parvulus (4B) at −20 and −80°C came very close and were higher than those at −196°C. When analysing the behaviour of each bacterium vs different freezing temperatures by means of the Kruskal–Wallis one-way ANOVA, no significant different medians of the survival rates at the three different temperatures were found with L. paracasei, P. parvulus, L. mali, L. hilgardii, L. mesenteroides and L. plantarum (Fig. 4: A, B, C, D, F and G). Indeed, they were found only O. oeni and L. brevis (Fig. 4: E and H). In L. brevis, a significant

Figure 2 The boxplots that result from the Kruskal–Wallis one-way ANOVA test considering the yeast survival percentage (Y-axis) to be the independent variable and yeast strain the grouping factor. (a) −20; (b) −80; (c) −196°C. A: Pichia membranifaciens; B: Starmerella bacillaris; C: Metschnikowia pulcherrima; D: Torulaspora delbrueckii; E: Hanseniaspora uvarum; F: Wickerhamomyces anomalus; G: Pichia kudriavzevi; H: Saccharomyces cerevisiae; I: Issatchenkia occidentalis; J: Schizosaccharomyces pombe; K: Dekkera bruxellensis. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 3 The boxplot that results from the Kruskal–Wallis one-way ANOVA test considering yeast survival percentage to be the independent variable and freezing temperature the grouping factor. Asterisk and circle label extreme data. [Colour figure can be viewed at wileyonlinelibrary.com]
Table 3 Survival percentages obtained after freeze-drying of LAB at different temperatures

| LAB strain               | Counts before freezing* (±SD) | Survival percentage (±SD) | –20°C     | –80°C     | –196°C    |
|--------------------------|-------------------------------|---------------------------|-----------|-----------|-----------|
| *Counts of the concentrated cell suspension. |
| **Pediococcus parvulus** | 1.2 × 10^11 (±1.4 × 10^10)   |                          | 4.2 × 10^10 (±2.9 × 10^10) | 4.1 × 10^11 (±3.3 × 10^10) | 3.1 × 10^10 (±3.9 × 10^10) |
| **Lactobacillus mali**   | 5.4 × 10^10 (±7.1 × 10^9)    |                          | 2.6 × 10^10 (±2.5 × 10^9)  | 1.9 × 10^11 (±1.5 × 10^10) | 5.1 × 10^10 (±1.4 × 10^10) |
| **Oenococcus oeni**      | 3.2 × 10^10 (±1.4 × 10^9)    |                          | 2.1 × 10^11 (±4.1 × 10^9)  | 1.2 × 10^11 (±4.0 × 10^10) | 7.3 × 10^10 (±0.3 × 10^10) |
| **Leuconostoc mesenteroides** | 3.2 × 10^10 (±7.1 × 10^9) |                          | 1.3 × 10^11 (±1.5 × 10^10) | 1.4 × 10^10 (±2.1 × 10^10) | 1.7 × 10^11 (±4.8 × 10^10) |
| **Lactobacillus plantarum** | 5.3 × 10^10 (±2.1 × 10^9)  |                          | 8.2 × 10^10 (±0.8 × 10^10) | 9.5 × 10^10 (±2.6 × 10^10) | 8.0 × 10^10 (±1.8 × 10^10) |
| **Lactobacillus brevis**  | 3.4 × 10^10 (±5.0 × 10^9)   |                          | 2.3 × 10^10 (±0.9 × 10^9)  | 1.4 × 10^10 (±0.2 × 10^10) | 1.2 × 10^11 (±6.3 × 10^10) |
| **Average**              | 1.4 × 10^10 (±4.0 × 10^9)   |                          | 2.1 × 10^10 (±2.0 × 10^9)† | 1.8 × 10^10 (±5.0 × 10^9)† | 2.3 × 10^10 (±4.7 × 10^9)§ |

*Counts of the concentrated cell suspension.
†Average of the survival rates for all the LAB obtained at –20°C.
‡Average of the survival rates obtained for all the LAB at –80°C.
§Average of the survival rates obtained for all the LAB at –196°C.

Figure 4 The boxplots that result from the Kruskal–Wallis one-way ANOVA test considering the survival percentage (Y-axis) of each LAB to be the independent variable and freezing temperature the grouping factor. A: Lactobacillus paracasei; B: Pediococcus parvulus; C: Lactobacillus mali; D: Lactobacillus hilgardii; E: Oenococcus oeni; F: Leuconostoc mesenteroides; G: Lactobacillus plantarum; H: Lactobacillus brevis. [Colour figure can be viewed at wileyonlinelibrary.com]

difference was found between the medians of the survival rates at –80 and –196°C (P = 0.022), and also between those that corresponded to –20 and –196°C in O. oeni (P = 0.005).

The medians of the survival rates of the different LAB at –20, –80 and –196°C (Fig. 5) showed significant differences (P = 0.008, P = 0.012 and P = 0.006 respectively). This meant that for each freezing temperature, the survival rate was affected by bacterium type. However, the pair-wise analysis showed only significant differences in the medians of these couples: L. brevis–L. paracasei, at –20°C (P = 0.008), and L. brevis–L. paracasei (P = 0.042) and L. brevis–P. parvulus at –80°C (P = 0.018).

When considering the medians of the survival rates of all the LAB vs the freezing temperatures together (Fig. 6), the Kruskal–Wallis one-way ANOVA analysis indicated that the medians of survival rates were not significantly affected by temperature (P > 0.695).
To summarize, LAB survival after freeze-drying was affected by only bacteria type, but not by freezing treatment.

In order to know whether cell morphology influenced the response of LAB vs freezing temperatures, LAB were grouped as rod (all *Lactobacillus* species) or coccus (*L. mesenteroides*, *P. parvulus* and *O. oeni*) cells. The survival rates of these two bacteria groups were analysed at −20, −80 and −196°C. The Kruskal–Wallis one-way ANOVA analysis showed no significant differences among the medians of the survival rates of the different cell morphologies for any freezing temperature (P > 0.05), although the most marked differences found between the survival of rod and coccus cells were found at −196°C (Fig. S1). At this temperature, rod cells seemed more resistant to freeze-drying than coccus cells. The analysis concluded that cell morphology did not influence the survival rate of bacteria at any freezing temperature.

Comparison of yeasts and LAB behaviours at different freezing treatments

In order to know whether the same freeze-drying procedure could be used to preserve yeasts and LAB from wine, the survival rates of the two sets of microorganisms were analysed when facing the tested freezing temperatures.

The survival of the yeast and LAB groups at the three freezing temperatures produced major differences in the response to freeze-drying (Tables 2 and 3). Higher survival rates were obtained for yeasts at −20 and −80°C (averages of 9.5 and 7.1% respectively), than at −196°C (an average of 0.4%), although the differences found for individual species were much more marked in some cases (Table 2). On the contrary, the LAB survival rates at −20, −80 and −196°C did not differ that much (averages of 21, 18 and 23% respectively; Table 3). The statistical analysis showed significantly different medians of the survival rates between these two groups of microorganisms at −20, −80 and −196°C (P = 0.000, in all cases), which means that yeasts and LAB behave differently when frozen at these temperatures (Fig. S2).

**Discussion**

Among the factors that determine post-freeze-drying survival rates, micro-organism type is one of the most important intrinsic factors, and the freezing protocol is one of the most relevant extrinsic elements (Tsvetkov and Shishkova 1982; Santivarangkna et al. 2008). We demonstrated that yeast species and freezing temperature are crucial factors for determining freeze-drying performance.
in yeasts. Major differences among the survival rates of different yeasts were found at each tested freezing temperature. There are several factors that may result in a different sensitivity of yeasts to freeze-drying: osmotolerance, size, cell wall, membrane composition, etc. Miyamoto-Shinohara et al. (2010) found a relationship between osmotolerance of yeasts and tolerance to freeze-drying. We observed that the strains of species P. membranifaciens, M. pulcherrima, T. delbrueckii, H. uvarum, P. kudriavzevii and W. anomalus, all of which have been reported as being osmotolerant (Wang et al. 2015), were more resistant at freeze-drying than the other yeasts. Although Wang et al. (2015) described S. cerevisiae as a species capable of resisting high sugar concentrations, Tofalo et al. (2009) described osmotolerance as a strain-dependent character in this species. As we did, other authors have also reported very low survival rates to freeze-drying for S. cerevisiae strains, especially when freezing is performed by dipping in liquid nitrogen (−196°C) (Wellman and Stewart 1973; Miyamoto-Shinohara et al. 2006). Hernández-López et al. (2003) tested the abilities of osmotolerant T. delbrueckii to survive, and to leave sweet and frozen sweet dough. These authors found that T. delbrueckii was more tolerant to freezing and to overcoming osmotic stress than S. cerevisiae strains. They concluded that the survival differences between these two yeasts were related to poor invertase activity, a low trehalose mobilization rate and rapid adaptation to the high osmotic pressure that T. delbrueckii exhibits. As Miyamoto-Shinohara et al. (2010) also pointed out, we observed that yeast size could be another reason to explain the differences in survival in the yeast group: the larger the yeast was, the lower the recorded survival. So P. membranifaciens, S. bacillaris, M. pulcherrima and T. delbrueckii (with a size range of 1.8–6 × 3–17 μm) were more resistant to freeze-drying than larger yeasts H. uvarum, S. pombe or D. bruxellensis (size range of 1.5–7 × 2.5–28 μm); the sizes of these yeasts species were obtained from Kurtzman and Fell (1998). As pointed by other authors, cell wall and membrane composition strongly affects yeast survival after freeze-drying (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 mannann, alkali-soluble glucan, alkali-insoluble glucan and small amounts of chitin (Nguyen et al. 1998). Most protein is covalently linked to mannan in the compounds named mannoprotein. The proportions of the different cell wall and membrane components vary with species and strongly influence the rheological properties of cell wall and membrane organization (Nguyen et al. 1998), which can influence survival rates to freezing and freeze-drying. Miyamoto-Shinohara et al. (2010) stated that the larger the amount of glucan in yeast walls, the higher the survival rate is after freeze-drying. However, glucan may also trap moisture, which can lower survival during storage. Moisture retention also occurs when yeasts are able to produce the extracellular polysaccharides that have the same effect as glucans (Miyamoto-Shinohara et al. 2010). We observed that the survival rate of P. kudriavzevii (syn. Issatchenkia orientalis) was 100 and 300 times higher than for L. occidentalis and S. cerevisiae. Similar differences between S. cerevisiae and L. orientalis have been reported by Miyamoto-Shinohara et al. (2010). It is generally accepted that freezing in liquid nitrogen (−196°C) is the method that results 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). This is because the cooling rate at this temperature, 300°C min−1 from Uzunova-Doneva and Donev (2000–2002), is too fast to allow internal water to migrate outside the cell, and the water frozen inside the cell results in lethal damage (Abadias et al. 2001). When comparing the yeast survival rates obtained from different preservation methods, the survival of freeze-dried yeast is lower than that obtained from freezing, i-drying or drying (Atkin et al. 1949; Miyamoto-Shinohara et al. 2010; Prakash et al. 2013). Russell and Stewart (1981) also demonstrated that the survival rates of three brewing yeasts, S. cerevisiae, Saccharomyces uvarum and Saccharomyces diastaticus, were higher when frozen than when freeze-dried.

The freeze-drying survival rates of LAB also differed between species, and between freezing treatments, but differences were much less marked than in yeasts. We did not notice a size effect on LAB survival as we did in yeast, possibly because the differences in size among bacteria were very small (from 0.5 to 1 μm diameter for O. oeni, P. cerevisiae and L. mesenteroides cells to 0.5–1 × 1.2–8 μm for L. brevis, L. hilgardii, L. mali, L. paracasei and L. plantarum) (Dicks and Holzapfel 2009; Hammes and Hertel 2009; Holzapfel et al. 2009a,b). Cell morphology did not explain the survival rate differences obtained with our results. Differences in peptidoglycan composition could, at least in part, explain the differences in the response to freezing temperatures that we found in the freeze-dried LABs. Thus, the bacteria with mesodiaminopimelic in their cell walls (L. paracasei and L. mali) significantly better tolerated freezing at −196°C than at −80°C. However, the cells with Lys-D-Asp (L. brevis, L. hilgardii, L. plantarum and P. parvalus) in their peptidoglycan molecules showed no significant differences in survival at the three freezing temperatures. The cell wall composition data were obtained from Bergey’s Manual of Systematic Bacteriology (Dicks and Holzapfel 2009;
Hammes and Hertel 2009; Holzapfel et al. 2009a,b). Zhao and Zhang found that malolactic species O. oeni and L. brevis displayed wider viability after freeze-drying when frozen at −65°C than at −20°C (Zhao and Zhang 2005, 2009). Yet in our case, the survival of these two species was greater at −20°C than at −80°C, although the differences in the cell survival rates that corresponded to these two temperatures were too small to be considered significant. The results for L. plantarum provided slightly higher survival rates at −80°C than at the other two temperatures, but these differences were not significant. We obtained survival values of 8–9% for L. plantarum at all three tested temperatures, which are lower than those reported by other authors (de Valdez et al. 1985; G-Alegria et al. 2004). The use of different lyoprotectors and rehydration media can explain these differences.

This work focused on the freeze-drying effect on microorganisms related to the winemaking process. We found major differences in the survival of yeasts and LAB: yeasts were more sensitive to this preservation method than bacteria, as previously demonstrated by Miyamoto-Shinohara et al. (2006) and Dumont et al. (2004). Dumont et al. (2004) reported similar findings when they tested the effect of different freezing rates on the survival of yeasts S. cerevisiae and Candida utilis, and bacterium L. plantarum. It is noteworthy that freezing at −196°C provided the best survival rates in bacteria, but the worst ones in yeasts. This differential fact could be related to the surface/volume (S/V) ratio, which determines thermal and water flow during freezing. Bacterial cells are smaller than yeast cells, and have a fivefold higher S/V ratio. Hence, water and heat will flow out faster from bacteria to thus prevent intracellular crystallization, which results in higher viability rates at this temperature. The cell wall and plasmatic membrane differences between bacteria and yeast species can also account for differences in survival (Dumont et al. 2004; Miyamoto-Shinohara et al. 2010). What clearly comes over from the literature is that freeze-drying has a more negative effect than freezing both yeast and bacteria, as formerly demonstrated by (Russell and Stewart 1981; Maicas et al. 2000); Bravo-Ferrada et al. (2015)). This is not surprising because they suffer not only the deleterious effects of freezing but also those that derive from vacuum drying. This latter process exposes cell envelopes to a hydrophobic environment that alters membrane permeability more drastically than freezing. We found marked differences in the abilities to survive among not only yeasts and bacteria but also among the different species that belong to both these groups. Although several authors have found that even different strains of the same species can respond differently to the same preservation method (Prakash et al. 2013; Bravo-Ferrada et al. 2015) the homogeneous behaviour of the yeast and LAB groups corroborates that the intraspecific differences in survival rates must be minor compared to interspecific ones. The results from this work evidence that differences in the survival of the distinct species must be related to differences in the morphology, cell envelope composition and metabolism of micro-organisms, which agree with several authors (Dumont et al. 2004; Miyamoto-Shinohara et al. 2008; Santivarangkna et al. 2008; Miyamoto-Shinohara et al. 2010; Prakash et al. 2013), and not by extrinsic factors (cooling rates, lyoprotectant and rehydration media), which were common for all the members of each microbial group.

Although liquid fresh or frozen yeast starters provide more viable cell populations, the short time frame of fresh cultures or the difficulties to distribute and store frozen yeast, justify that the majority of yeast starter cultures used for food fermentations are sold as active dried yeasts (Aguilera and Karel 1997; Krieger-Weber 2009). Nonetheless, a few yeasts are prepared in a freeze-dried form for pharmaceutical, purposes (probiotics), or as food additives or starters (Kelesidis and Pothoulakis 2012). LAB-dried starters are practically inexistent in the market, which is possibly due to the low tolerance of these bacteria to drying. Although not many works have compared the effects of drying and freeze-drying, Kim and Bhowmik (1990) found that Streptococcus thermophilus survived less in spray-dried yogurt powder than in freeze-dried powder. Besides greater storage stability, easy handling during storage, distribution and application, this fact makes freeze-drying preferable to freezing to produce commercial starters, instead of frozen cultures with a higher percentage of viable cells and cells that require less time for activation (Buckenhuskes 1993).

From the results obtained herein, we recommend that freezing prior to freeze-drying should be performed at −20°C for yeasts and for bacteria O. oeni and P. parvulus, and at −196°C for other LAB, in order to achieve the best survival rates.

The data obtained in our experiments are important from a practical point of view as optimal freezing temperatures to perform freeze-drying of wine micro-organisms can be deduced. Freezing at −20°C ensures yeast survival that is 10 to 1.8 × 10⁴ higher than at −196°C, whereas this last temperature ensures only 1–5 times more LAB survival than at −20°C. Optimizing freeze-drying conditions ensures better performance to preserve micro-organisms of industrial importance in private and public culture collections, and helps to define guidelines to improve the production process of commercial starter cultures.

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Conflict of Interest
No conflict of interest is declared.

REFERENCES

Abadias, M., Benabarre, A., Teixidó, N., Usall, J. and Viñas, I. (2001) Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake. Int J Food Microbiol 65, 173–182.

Aguilera, J.M. and Karel, M. (1997) Preservation of biological materials under desiccation. Crit Rev Food Sci Nutr 37, 287–309.

Atkin, L., Moses, W. and Gray, P.P. (1949) The preservation of yeast cultures by lyophilization. J Bacteriol 6, 575–578.

Berbegal, C. (2014) Novel liquid starter cultures for malolactic fermentation in wine. PhD Thesis, University of Valencia, Spain.

Berbegal, C., Benavent-Gil, Y., Navascués, E., Calvo, A., Albors, C., Pardo, I. and Ferrer, S. (2017) Lowering histamine formation in a red Ribera del Duero wine (Spain) by using an indigenous O. oeni strain as a malolactic starter. Int J Food Microbiol 244, 11–18.

Bravo-Ferrada, B.M., Brizuela, N., Gerbino, E., Gómez-Zavaglia, A., Semorile, L. and Tymczyszyn, E.E. (2015) Effect of protective agents and previous acclimation on ethanol resistance of frozen and freeze-dried Lactobacillus plantarum strains. Cryobiology 71, 522–528.

Buckenhüüs, H.J. (1993) Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol Rev 12, 253–272.

Caplice, E. and Fitzgerald, G.F. (1999) Food fermentations: role of microorganisms in food production and preservation. Int J Food Microbiol 50, 131–149.

Dicks, L.M.T. and Holzapfel, W.H. (2009) Genus Oenococcus. In Bergey's Manual of Systematic Bacteriology ed. De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H. and Whitman, W., pp. 635–642. Springer: New York, USA.

Donev, T., Savova, I. and Kujumdzieva, A. (1995) Influence of the cultivation conditions on yeast strains survival after lyophilization. J Cult Collect 1, 38–41.

Dumont, F., Marechal, P.-A. and Gervais, P. (2004) Cell size and water permeability as determining factors for cell viability after freezing at different cooling rates. Appl Environ Microbiol 70, 268–272.

G-Alegria, E., Lopez, I., Ruiz, J.I., Saenz, J., Fernandez, E., Zarazaga, M., Dízy, M., Torres, C. et al. (2004) High tolerance of wild Lactobacillus plantarum and Oenococcus oeni strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol Lett 230, 53–61.

Hammes, W.P. and Hertel, C. (2009) Genus Lactobacillus. In Bergey's Manual of Systematic Bacteriology ed. De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H. and Whitman, W., pp. 465–511. Springer: New York, USA.

Hernández-López, M.J., Prieto, J.A. and Rández-Gil, F. (2003) Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between Torulaspora delbrueckii and Saccharomyces cerevisiae baker's yeast strains. Antonie Van Leeuwenhoek 84, 125–134.

Hsier, E., Janzen, T., Henriksen, C.M., Rattray, F., Brockmann, E. and Johansen, E. (1999) The production, application and action of lactic cheese starter cultures. In Technology of Cheesemaking ed. Law, B.A., pp. 99–131. Sheffield: Sheffield Academic Press.

Holzapfel, W.H. (2002) Appropriate starter culture technologies for small-scale fermentation in developing countries. Int J Food Microbiol 75, 197–212.

Holzapfel, W., Franz, C.M.A.P., Wolfgang, L. and Dick, L. (2009a) Genus Pediococcus. In Bergey's Manual of Systematic Bacteriology ed. De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H. and Whitman, W., pp. 513–520: Springer: New York, USA.

Holzapfel, W.H., Bjorkroth, J.A. and Dicks, L.M.T. (2009b) Genus Leuconostoc. In Bergey's Manual of Systematic Bacteriology ed. De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H. and Whitman, W., pp. 624–635. Springer: New York, USA.

Kelesidis, T. and Pothoulakis, C. (2012) Efficacy and safety of the probiotic Saccharomyces boulardii for the prevention and therapy of gastrointestinal disorders. Therap Adv Gastroenterol 5, 111–125.

Kim, S.S. and Bhowmik, S.R. (1990) Survival of lactic acid bacteria during spray drying of plain yogurt. J Food Sci 55, 1008–1010.

Krieger, S. (2009) Application of yeast and bacteria as starter culture. In Biology of Microorganisms on Grapes, in must and in Wine ed. Köning, H., Unden, G. and Fröhlich, J., pp. 489–511. Berlin: Springer.

Krieger-Weber, S. (2009) Application of yeast and bacteria as starter cultures. In Biology of Microorganisms on Grapes, in must and in Wine ed. Köning, H., Unden, G. and Fröhlich, J., pp. 489–511. Berlin: Springer-Verlag.

Kumar, G.P., Prashanth, N. and Kumari, B.C. (2011) Study of the probiotic yeast cultures by lyophilization. J Cult Collect 642. Springer: New York, USA.

Kurtzman, C. and Fell, J.W. (1998) The Yeast. A Taxonomic Study. Amsterdam, The Netherlands: Elsevier.

Lucio, O., Pardo, I., Krieger-Weber, S., Heras, J.M. and Ferrer, S. (2016) Selection of Lactobacillus strains to induce freezing temperature effect on cell survival
biological acidification in low acidity wines. *LWT – Food Sci Technol* **73**, 334–341.

Maicas, S., Pardo, I. and Ferrer, S. (2000) The effects of freezing and freeze-drying of *Oenococcus oeni* upon induction of malolactic fermentation in red wine. *Int J Food Sci Technol* **35**, 75–79.

Miyamoto-Shinohara, Y., Imaizumi, T., Sukenobe, J., Murakami, Y., Kawamura, S. and Komatsu, Y. (2000) Survival rate of microbes after freeze-drying and long-term storage. *Cryobiology* **41**, 251–255.

Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T. and Nakahara, T. (2006) Survival curves for microbial species stored by freeze-drying. *Cryobiology* **52**, 27–32.

Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T. and Nakahara, T. (2008) Survival of freeze-dried bacteria. *J Gen Appl Microbiol* **54**, 9–24.

Miyamoto-Shinohara, Y., Nozawa, F., Sukenobe, J. and Imaizumi, T. (2010) Survival of yeasts stored after freeze-drying or liquid-drying. *J Gen Appl Microbiol* **56**, 107–119.

Morgan, C.A., Herman, N., White, P.A. and Vesey, G. (2006) Preservation of micro-organisms by drying: a review. *J Microbiol Methods* **66**, 183–193.

Nakamura, T., Takagi, H. and Shima, J. (2009) Effects of ice-seeding temperature and intracellular trehalose contents on survival of frozen *Saccharomyces cerevisiae* cells. *Cryobiology* **58**, 170–174.

Nguyen, H.T., Fleet, H.G. and Rogers, L.P. (1998) Composition of the cell walls of several yeast species. *Appl Microbiol Biotechnol* **50**, 206–212.

Pekkonen, K.S., Roos, Y.H., Miao, S., Ross, R.P. and Stanton, C. (2008) State transitions and physicochemical aspects of cryoprotection and stabilization in freeze-drying of *Lactobacillus rhamnosus* GG (LGG). *J Appl Microbiol* **104**, 1732–1743.

Peiren, J., Buyse, J., de Vos, P., Lang, E., Clermont, D., Hamon, S., Bégaud, E., Bizet, C. et al. (2015) Improving survival and storage stability of bacteria recalcitrant to freeze-drying: a coordinated study by European culture collections. *Appl Microbiol Biotechnol* **99**, 3559–3571.

Prakash, O., Nimonkar, Y. and Shouche, Y.S. (2013) Practice and prospects of microbial preservation. *FEMS Microbiol Lett* **339**, 1–9.

Russell, I. and Stewart, G.G. (1981) Liquid nitrogen storage of yeast cultures compared to more traditional storage methods. *J Am Soc Brew Chem* **39**, 19–24.

Santivarangkna, C., Kulazik, U. and Foerst, P. (2008) Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. *J Appl Microbiol* **105**, 1–13.

Seki, S., Kleinhaus, F.W. and Mazur, P. (2009) Intracellular ice formation in yeast cells vs. cooling rate: predictions from modeling vs. experimental observations by differential scanning calorimetry. *Cryobiology* **58**, 157–165.

Tofal, R., Chaves-López, C., di Fabio, F., Schirome, M., Felis, G.E., Torriani, S., Paparella, A. and Suzuki, G. (2009) Molecular identification and osmotolerant profile of wine yeasts that ferment a high sugar grape must. *Int J Food Microbiol* **130**, 179–187.

Tsvetkov, T. and Shishkova, I. (1982) Studies on the effects of low temperatures on lactic acid bacteria. *Cryobiology* **19**, 211–214.

Tymczyzszyn, E.E., del Rosario Diaz, M., Gomez-Zavaglia, A. and Disalvo, E.A. (2007) Volume recovery, surface properties and membrane integrity of *Lactobacillus delbrueckii* subsp. bulgaricus dehydrated in the presence of trehalose or sucrose. *J Appl Microbiol* **103**, 2410–2419.

Uzunova-Donева, T. and Donев, T. (2000–2002) Influence of the freezing rate on the survival of strains *Saccharomyces cerevisiae* after cryogenic preservation. *J Cult Collect* **3**, 78–83.

de Valdez, G.F., de Giori, G.S., de Ruiz Holgado, A.P. and Oliver, G. (1985) Effect of the rehydration medium on the recovery of freeze-dried lactic acid bacteria. *Appl Environ Microbiol* **50**, 1339–1341.

Wang, H., Hu, Z., Long, F., Niu, C., Yuan, Y. and Yue, T. (2015) Characterization of osmotolerant yeasts and yeast-like molds from apple orchards and apple juice processing plants in China and investigation of their spoilage potential. *J Food Sci* **80**, M1850–M1860.

Wellman, A.M. and Stewart, G.G. (1973) Storage of brewing yeasts by liquid nitrogen refrigeration. *Appl Microbiol* **26**, 577–583.

Zhao, G. and Zhang, G. (2005) Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *J Appl Microbiol* **99**, 333–338.

Zhao, G. and Zhang, G. (2009) Influence of freeze-drying conditions on survival of *Oenococcus oeni* for malolactic fermentation. *Int J Food Microbiol* **135**, 64–67.

Zúñiga, M., Pardo, I. and Ferrer, S. (1993) An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. *Int J Food Microbiol* **18**, 37–42.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** The boxplots that result from the Kruskal–Wallis one-way ANOVA test considering LAB survival percentage to be the independent variable and LAB cell shape the grouping factor. (a) −20; (b) −80; (c) −196°C. 1: rod shape; 2: coccius shape.

**Figure S2** The boxplots that result from the Kruskal–Wallis one-way ANOVA test considering survival percentage to be the independent variable and freezing temperature the grouping factor. (a) −20; (b) −80; (c) −196°C. 1: set of all yeasts, 2: set of all the lactic acid bacteria.