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Article

Probiotic Rosé Wine Made with *Saccharomyces Cerevisiae* var. *Boulardii*

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Abstract: This paper reports for the first time on the production of probiotic alcoholic and non-alcoholic rosé wines with enhanced health benefits made with *Saccharomyces cerevisiae* var. *boulardii* probiotic yeast. The alcohol, sugar, volatile acidity lactic and malic acid contents were assessed for *S. cerevisiae* var. *boulardii* before and after fermentation and distillation and compared with a conventional *Saccharomyces cerevisiae* (ex-*bayanus*) yeast. The free amino nitrogen and gluconic acid concentrations in the musts were determined. Yeast viability was evaluated after fermentation and distillation as a function of time (0, 15 days, 3 months and 6 months) both at room temperature (25±0.5ºC) and refrigerator temperature (4±0.5ºC). The results obtained showed that the probiotic rosé wine produced with *S. cerevisiae* var. *boulardii* possesses the typical values and sensory attributes of other commercial wines produced with *S. cerevisiae* (ex-*bayanus*). The probiotic *S. cerevisiae* var. *boulardii* yeast survives the high alcohol content produced during fermentation and vacuum distillation. The study also showed that this probiotic rosé wine stored either at room temperature or in a refrigerator keeps its probiotic viability for at least six months, which makes it a promising for large-scale production, in which long storage times are required by both producers and consumers.

Keywords: rosé wine; probiotic yeast; fermentation; distillation; viability

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1. Introduction

Although many different varieties of bacteria and yeasts can be used in winemaking (Grangeteau et al., 2017), the greatest diversity of microorganisms can be found in the unfermented must, such as *Saccharomyces, Candida, Hanseniaspora and Cryptococcus*, among others. Most of these species cannot survive in alcohol concentrations above 5% ethanol, at which point *Saccharomyces* yeasts remain alive as the dominant ones in the medium. Non-*Saccharomyces* yeasts only metabolize during the first days of fermentation, although their effect can still contribute significantly to the wine's aroma (Amerine and Kunker, 1968). In fact, one of the latest trends in winemaking consists of inoculating a non-*Saccharomyces* yeast, followed by inoculation with a common industrial yeast that can survive the high alcohol concentrations in the last stage of fermentation (Dutraive et al., 2019). In this context, sequential fermentations can meet the growing demand for new and improved wine yeast strains, adapted to different types and styles of wine (Padilla et al., 2016). *Saccharomyces cerevisiae* var. *boulardii* is a probiotic yeast that has shown a resistance to 6-8% ethanol similar to that of other common beer strains (Mulero-Cerezo et al., 2019; Ramírez-Cota et al., 2021). The temperature of fermenting red wine is usually from 22 to 28ºC, while that of white or rosé wines is usually around 18 to 20ºC (Amerine, 2020). Fermentation is mostly carried out by *Saccharomyces cerevisiae* (ex-*bayanus*), which are strains carefully selected for their better tolerance to alcohol and higher resistance to sulfuric acid and killer factors than other yeasts and bacteria (Masneuf-Pomarède et al., 2010).

In the wine regions in hot climates, yeasts with a high resistance to alcohol are becoming more and more important. Hot and dry summers can block phenolic ripening, forcing growers to harvest grapes when they are high in sugar and low in acidity (Mira de Orduña, 2010). Different types of unconventional yeasts have been suggested for sequential culture with wine yeasts to modulate acidity, an alternative to adding external tartaric acid. Using unconventional yeasts with a low sugar-to-ethanol conversion ratio has also been suggested to obtain wines with a low alcohol content (Ciani et al., 2016). *S. cerevisiae* var. *boulardii* is able to grow and colonize beer wort and raise the antioxidant capacity and acidity of the final product (Mulero-Cerezo et al., 2019), two useful characteristics that could help to partially supply metabisulfite and tartaric acid. The *S. cerevisiae* var. *boulardii* strains share the clade with wine *Saccharomyces cerevisiae* strains BC187, YJM1387, YJM1417, YJM1332 and R008, brewery strains *S. cerevisiae* YJM1477 and *S. cerevisiae* strain YJM1242 isolated from fruits (Khatri et al., 2017).

The optimal growth temperature of *S. cerevisiae* var. *boulardii* is 37°C, which makes it reach high concentrations in the gastrointestinal tract (GT) in a short period of time (Karen et al., 2010). It also possesses the capacity to inhibit the growth, adherence and invasion of multiple pathogens such as *Clostridium difficile* (Surawicz et al., 2000), *Escherichia coli* (Czerucka et al., 2000), and *Candida albicans* (Berg et al., 1993) both *in vitro* and *in vivo* conditions to the epithelial layer of the GT. Rosé wines possess high anthocyanin contents that vary from 35 to 160 mg L^{-1} (Murat et al., 2003). Anthocyanin has been shown to be antidiabetic, anticancer, anti-inflammatory, antimicrobial, and is non-fattening, as well as preventing cardiovascular diseases (Khoo et al., 2017).

In this study, we hypothesized that *S. cerevisiae* var. *boulardii* would be a valuable probiotic starter for non-alcoholic rosé winemaking, would be able to resist the high alcohol content after fermentation and would survive distillation even when stored for several months. The probiotic rosé wine thus produced has enhanced health benefits over other rosé wines produced by the common *S. cerevisiae* (ex-*bayanus*) yeast.

2. **Materials and methods.**

2.1. Materials

S. cerevisiae var. *boulardii*, CECT 1474, was purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain). *S. cerevisiae (*ex-*bayanus)* (EC-1118), Lalvin® yeast. was purchased from Lallemand (Montréal, Canada) These yeast strains were

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suspended in distilled water to be grown in Sabouraud Glucose Agar with Chloramphenicol (SGAC, Sigma-Aldrich, Steinheim, Switzerland) solid medium plates at their optimal culture temperature of 37°C for isolation. *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*) were then grown in Sabouraud broth (Scharlab, Sentmenat, Spain) with an orbital shaker-incubator for 24h, also at 37°C. 1mL aliquots of pure cultures were subsequently frozen at -80°C. Tartaric acid powder was purchased from MANUEL RIESGO, S.A, Madrid, Spain.

2.2. Wort preparation.

Monastrell variety grapes with organic certification were selected from the *Viñas Familia Gil* wine producers in Jumilla, Spain. The must was made in the same way as rosé wines are normally made, by pressing the grapes shortly after harvesting. The must was frozen at -20ºC and stored for future use. Before fermentation, the must was unfrozen and kept in a refrigerator at 4ºC to inhibit fermentation, while the sludge was allowed to settle for 24 hours. The sludge level was measured by a turbidity meter (HI 93703 Model, HANNA Instruments, Gipuzkoa, Spain). Settling took place after decanting and removing the lees by suction until the sludge reached a turbidity value of about 75 nephelometric turbidity units (NTU). All varieties of musts between 50 and 150 NTU do not show any significant differences in their enological parameters (Burin et al., 2015).

2.3. Bioreactor fermentation

Bioreactor fermentation was at 24±1°C in an autoclave (121° for 20 minutes) 2 L Biostat A bench-top bioreactor (Sartorius, Frankfurt, Germany) containing 1 L of autoclaved must. The must pH was adjusted to 3.5 by adding tartaric acid, the normal practice of wine producers. Bioreactor cultures were conducted in anaerobic conditions without stirring to ensure homogeneous mixing and low foam formation. Inoculation was by 0.5 mL of the yeast culture at an average concentration of 10^7 colony-forming units per mL (CFU/mL).

2.4. Specific gravity

The specific gravities of the wort (ρ_{wort}) and the fermented rosé wine (ϱ_{wine}) were determined by an EasyDens meter (Anton-Paar GmbH, Graz, Austria) as a function of time to detect the end of fermentation (no further change in specific gravity).

2.5. Yeast viability

The cell viability of the yeasts present in the fermented and distilled rosé wines was evaluated by the colony-counting method (Martí et al., 2018) as a function of time (0, 15 days, 3 months and 6 months) at room temperature (25±0.5ºC) and refrigerator temperature (4±0.5ºC) to determine the CFU/mL viable cells in each wine. Serial dilutions of the fermented and distilled wines were prepared in Sabouraud broth, after which 100 µL of each cell dilution was spread on SGAC plates and grown at 25°C for 48 hours. This experiment was performed three times to ensure reproducibility.

2.6. Wine distillation

The wine was distilled in a R-210 rotary evaporator (BÜCHI Labortechnik AG, Switzerland) under vacuum for three hours to ensure alcohol separation.

2.7. Wine parameters

Alcohol, sugars, volatile acidity, lactic, malic and gluconic acid were assessed for *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*) before and after fermentation and distillation by a WineScan™ analyzer from FOSS (Hillerød, Denmark) calibrated by an QKit™ 8 ready-made reference kit at the *Viñas Familia Gil* winery (Jumilla, Spain).

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2.8. Sensory Evaluation

After 10 days of fermentation and subsequent distillation a sensory evaluation was performed by a panel of four trained experts. The products were tested at 16ºC. The descriptive assessment of the wine's attributes (alcohol, aroma, fruitiness, sweetness, yeastiness, mustiness, citrus flavor and bitterness) was assessed on a scale of from 0 (extremely unpleasant) to 7 (extremely pleasant).

2.9. Statistical analysis

The statistical analysis was performed by ANOVA and followed by Tukey's post hoc test of the pH and specific gravity evolution during fermentation with both yeast strains and sensory evaluation results on GraphPad Prism 6 software at a significance level of at least $p < 0.05$.

3. Results and Discussion

Figure 1 shows a scheme of the wine fermentation and distillation process produced with probiotic *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*) as control.

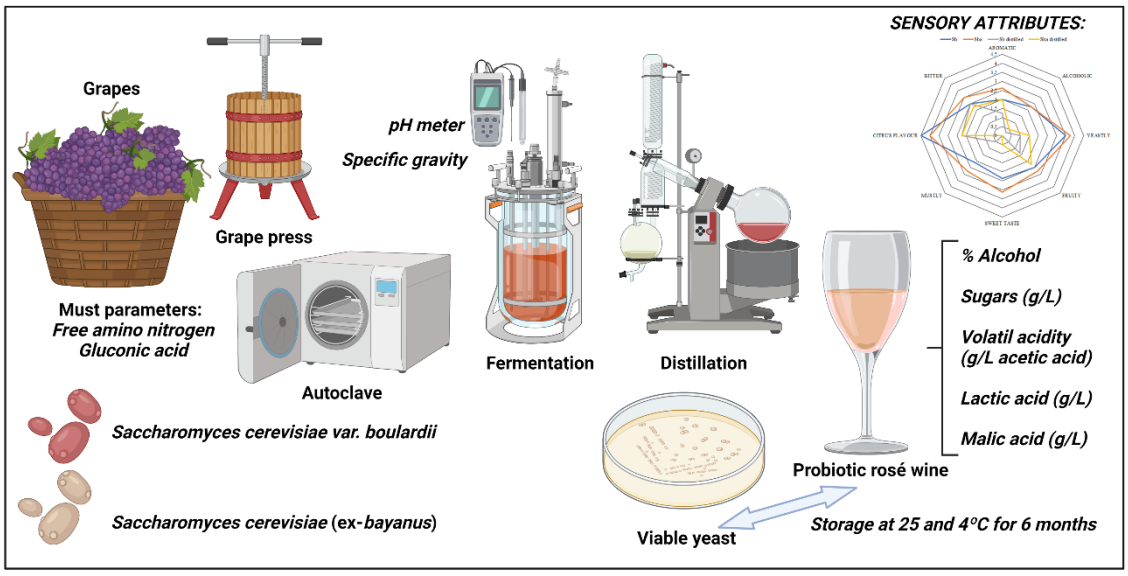

Figure 1. Scheme of the wine fermentation and distillation produced with probiotic *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*) as control.

3.1. Fermentation

Rosé wine was used in the tests as it does not need malolactic fermentation and is a more homogeneous medium since red wine is fermented along with the grape skins and seeds (Cerpa-Calderón and Kennedy, 2008). The correct fermentation temperature is essential as it depends not only on the type of wine to be fermented (red, white or rosé) but also the yeast used (Deed et al., 2017; Samoticha et al., 2019). Since our goal was to produce a rosé wine with viable *S. cerevisiae* var. *boulardii* cells after the aggressive alcohol formation process, the fermentation was carried out at 24±1°C. a temperature not very far from the optimal growth conditions of *S. cerevisiae* var. *boulardii* (37ºC (Karen et al., 2010)). This temperature also significantly reduces refrigeration costs when production is scaled up to an industrial level. Figure 2 shows specific gravity and pH evolution during fermentation of the rosé wine produced with *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*).

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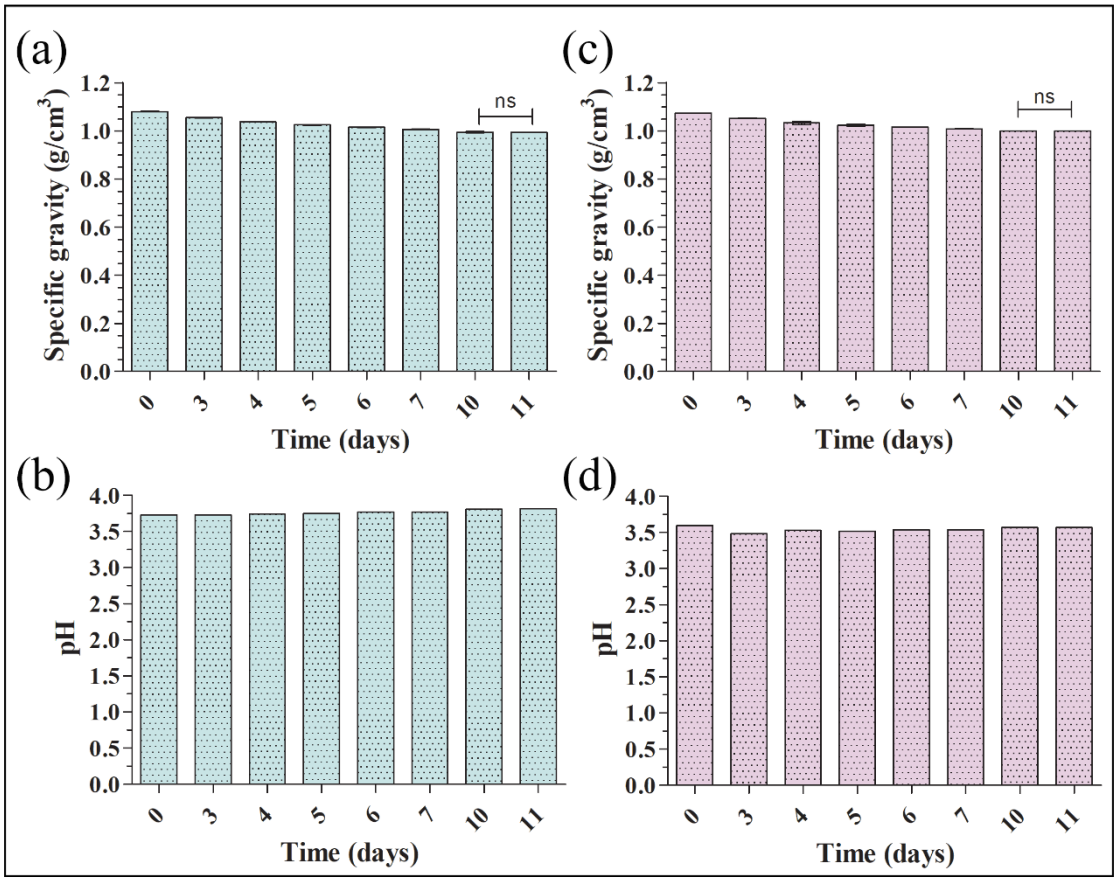

Figure 2. Specific gravity and pH evolution during fermentation for the rosé wine produced with *S. cerevisiae* var. *boulardii* (a&b, respectively) and with *S. cerevisiae* (ex-*bayanus*) (c&d, respectively). Statistical analysis performed by ANOVA, followed by Tukey's post hoc test at a significance level of at least $p < 0.05$. ns: not significant.

After 10 days both yeasts finalized fermentation with no further significant changes in their specific gravity and thus no additional generation of ethanol from sugars. Figure 2 also shows that pH hardly changes during fermentation by the commercial *S. cerevisiae* (ex-*bayanus*) or probiotic *S. cerevisiae* var. *boulardii*.

3.2. Rosé wine parameters

Table 1 shows the parameter values (alcohol, sugars, volatile acidity, free amino nitrogen, lactic and malic acid) determined for the must and rosé wines for *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*) before and after distillation, also the free amino nitrogen and gluconic acid concentrations of the musts.

Table 1. Must and rosé wine parameters: alcohol, sugars, volatile acidity, lactic and malic acid for *S. cerevisiae* var. *boulardii* (*Sb*) and *S. cerevisiae* (ex-*bayanus*) (*Sba*) before and after fermentation and distillation. Free amino nitrogen (FAN) and gluconic acid concentrations of the musts.

Parameters	**Must used with *Sb***	**Rosé wine (*Sb*)**	**Distilled rosé wine (*Sb*)**	**Must used with *Sba***	**Rosé wine (*Sba*)**	**Distilled rosé wine (*Sba*)**
Alcohol (% Vol)	0	11.06	0.64	0	9.54	1.48
Sugars (g/L)	189.78	0.97	0.36	167.48	1.01	0.14

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Volatile acidity (g/L acetic acid)	0.09	0.3	0.4	0.22	0.23	0.36
Free amino nitrogen, FAN (g/L)	125	-	-	133	-	-
Lactic acid (g/L)	0	0.01	0	0	0	0
Malic acid (g/L)	1.10	1.19	1.58	1.19	1.04	1.74
Gluconic acid (g/L)	1.07	-	-	2.11	-	-

The differences between the two musts used suggest that the one used for *S. cerevisiae* (ex-*bayanus*) suffered some type of bacterial growth before being autoclaved, due to the lower initial sugar levels, a higher volatile acidity indicator of bacterial growth, lower FAN and higher gluconic acid, which is a good indicator of fungal growth. Gluconic acid only indicates the quality of the grapes, so that this parameter was only measured for the musts. The ethanol concentrations in both rosé wines were as expected according to the initial sugar level 11.6% (*S. cerevisiae* var. *boulardii*) and 9.56 (*S. cerevisiae* (ex-*bayanus*)). After distillation a residual ethanol percentage of 0.64 and 1.48 in the rosé wines fermented with *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*) were obtained, respectively. However, with the latest efficient methods of dealcoholizing wine and beer it would not be difficult to obtain 0.0% vol.

Both yeasts fermented the must until leaving a residual sugar of approximately 1 gr/L, which produces wines considered dry as they have less than 4 gr/L (Takaya et al., 2002). The wine industry has recently focused on experimenting with non-saccharomyces yeasts, such as (*Torulaspora delbrueckii*, *Pichia kluyveri*, *Lachancea thermotolerans, Candida pulcherrima* and *Metschnikowia pulcherrima*). These are used to start fermentation and obtain the characteristic organoleptic wine profiles but are unable to produce complete fermentation due to their low tolerance to alcohol (Jolly et al., 2014). Unlike these yeasts, our studies indicate that *S. cerevisiae* var. *boulardii* could be used as the single wine fermentation agent since it tolerates alcohol well and, in this aspect, meets the desired parameters for large scale wine production.

Acetic acid is the main component that influences volatile acidity and can be generated during alcohol fermentation by yeast or bacteria (Vilela-Moura et al., 2011). Since the musts were autoclaved and the samples were analyzed just after fermentation under sterile conditions, fermentation by acetic acid bacteria can be ruled out, so that the values shown are presumably a product of yeast metabolism. Both rosé wines showed low volatile acidity, 0.3 g/L (*S. cerevisiae* var. *boulardii*) and 0.23 g/L (*S. cerevisiae* (ex-*bayanus*)), typical characteristics of the yeasts used in the wine industry. As regards this aspect both rosé wines therefore meet the desired parameters for commercial wine making.

Both FAN values are close to ~130 g/L, which is the optimal value proposed for some types of alcohol fermentation (Boro et al., 2022).

Lactic acid fermentation was not started in the present study as this type of fermentation is only desirable for red wines. A concentration of 0 g/L of lactic acid was thus determined in both rosé wines, indicating that there was no contamination during the fermentation process.

Malic acid is a product of the grape and is not obtained during fermentation. This is a desirable acid in both rosé and white wines as it provides a sensation of freshness. The malic acid concentration increased after distillation, showing similar values for both rosé wines.

S. cerevisiae var. *boulardii* thus shows a fermentative profile similar to the control yeast (*S. cerevisiae* (ex-*bayanus*)), indicating its suitability for large scale wine production.

3.3. Yeast viability

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The probiotic value of rosé wine depends on the amount of viable probiotic cells it contains after fermentation and distillation. Yeast viability was measured by the colony counting method. The yeast viability results of *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (ex-*bayanus*) before and after 15 days, 3 months and 6 months are shown in Table 2.

Table 2. Yeast viability of the wines produced with *S. cerevisiae* var. *boulardii* (*Sb*) and *S. cerevisiae* (ex-*bayanus*) (*Sba*) before and after distillation at ambient temperature (25ºC) and refrigerated at 4ºC in CFU/mL.

Temperature = 25ºC				
Time	***Sb* distilled**	***Sb***	***Sba* distilled**	***Sba***
0	$1.41 \cdot 10^7 \pm 1.91 \cdot 10^6$	$1.29 \cdot 10^7 \pm 1.13 \cdot 10^6$	$1.93 \cdot 10^7 \pm 6.08 \cdot 10^6$	$1.80 \cdot 10^7 \pm 8.49 \cdot 10^6$
15 days	$1.04 \cdot 10^7 \pm 1.13 \cdot 10^6$	$7.95 \cdot 10^6 \pm 6.36 \cdot 10^5$	$1.27 \cdot 10^6 \pm 9.90 \cdot 10^4$	$2.82 \cdot 10^7 \pm 2.29 \cdot 10^7$
3 months	$3.01 \cdot 10^5 \pm 5.87 \cdot 10^4$	$1.25 \cdot 10^6 \pm 7.07 \cdot 10^4$	$9.50 \cdot 10^5 \pm 2.12 \cdot 10^5$	$7.95 \cdot 10^5 \pm 1.34 \cdot 10^5$
6 months	$1.23 \cdot 10^6 \pm 7.78 \cdot 10^4$	$\mathbf{5.60 \cdot 10^3 \pm 5.66 \cdot 10^2}$	$1.09 \cdot 10^6 \pm 1.41 \cdot 10^4$	$\mathbf{1.67 \cdot 10^5 \pm 6.36 \cdot 10^4}$
Temperature = 4ºC				
Time	***Sb* distilled**	***Sb***	***Sba* distilled**	***Sba***
0	$2.51 \cdot 10^7 \pm 3.54 \cdot 10^5$	$2.97 \cdot 10^7 \pm 3.32 \cdot 10^6$	$1.93 \cdot 10^7 \pm 6.08 \cdot 10^6$	$1.80 \cdot 10^7 \pm 8.49 \cdot 10^6$
15 days	$1.94 \cdot 10^7 \pm 2.55 \cdot 10^6$	$9.85 \cdot 10^6 \pm 1.06 \cdot 10^6$	$2.56 \cdot 10^7 \pm 1.30 \cdot 10^7$	$2.10 \cdot 10^7 \pm 1.05 \cdot 10^7$
3 months	$6.05 \cdot 10^6 \pm 6.36 \cdot 10^5$	$2.68 \cdot 10^6 \pm 3.29 \cdot 10^6$	$7.30 \cdot 10^6 \pm 3.82 \cdot 10^6$	$7.20 \cdot 10^5 \pm 5.66 \cdot 10^4$
6 months	$2.10 \cdot 10^6 \pm 1.27 \cdot 10^6$	$\mathbf{7.45 \cdot 10^3 \pm 2.12 \cdot 10^2}$	$1.66 \cdot 10^6 \pm 4.81 \cdot 10^5$	$\mathbf{2.16 \cdot 10^5 \pm 3.39 \cdot 10^4}$

After 6 months of storage at 25ºC and 4ºC, both yeasts in the alcoholic and non-alcoholic wines were still viable with significant viable cell concentrations. However, yeast viability declined in both alcoholic wines, probably due to the presence of ethanol at both temperatures (see concentrations highlighted in bold in Table 2).

Figure 3 gives the representative plate images of the viable yeast colonies in the rosé wines with a dilution factor of 10^{-4} at room (25ºC) and refrigerated (4ºC) temperatures.

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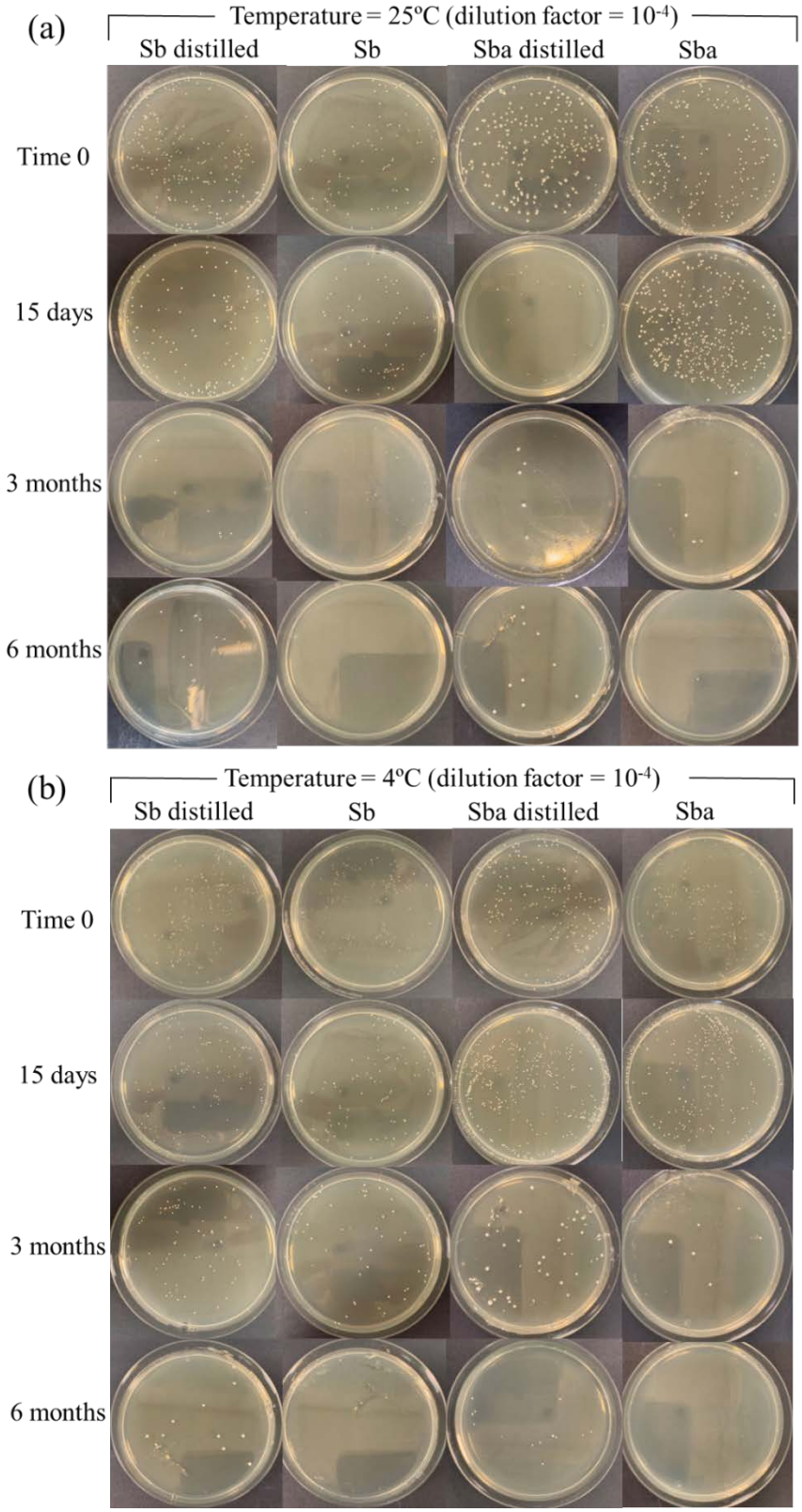

Figure 3. Representative plate images of the viable yeast colonies in the rosé wines produced with *S. cerevisiae* var. *boulardii* (*Sb*) and *S. cerevisiae* (ex-*bayanus*) (*Sba*) before and after distillation with a dilution factor of 10^{-4} at (a) room temperature (25ºC) and at (b) refrigerated temperature (4ºC) by the colony counting method.

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3.4. Sensory evaluation

Although the analysis of the main volatile compounds in other probiotic drinks, such as beer produced with *S. cerevisiae* var. *boulardii* has shown no negative effects on beer aroma (Capece et al., 2018; Mulero-Cerezo et al., 2019), a comprehensive sensory evaluation of the probiotic rosé wine's attributes before and after distillation was performed here for the first time.

Figure 4 shows that both yeasts produced rosé wines with similar sensory attributes before and after distillation.

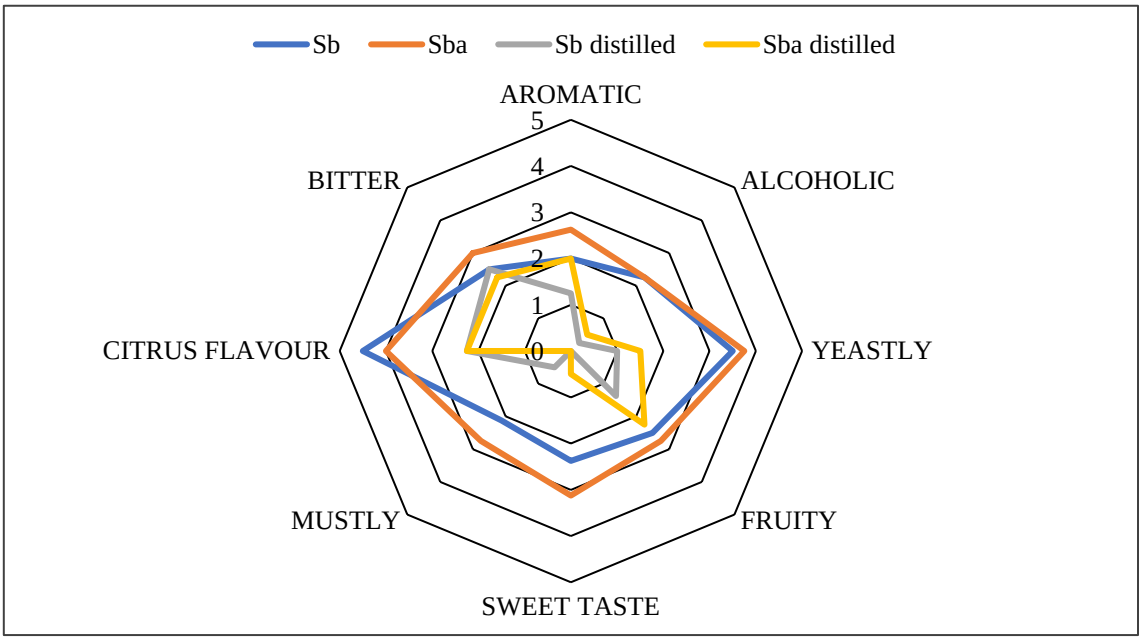

Figure 4. Results of the sensory evaluation of the rosé wines produced with the *S. cerevisiae* (ex-bayanus) and *S. cerevisiae* var. *boulardii* strains before and after distillation on a scale of from 0 (extremely unpleasant) to 7 (extremely pleasant). Results expressed as the mean of each sensory attribute: alcohol content, aroma, fruitiness, sweetness, yeastiness, mustiness, citric flavor, bitterness.

Of the alcoholic rosé wines, the probiotic wine was found to be more citric and had a sweeter taste, which are desirable qualities in white and rosé wines. However, a somewhat lower general aromatic intensity was found in these wines than in that produced with *S. cerevisiae* (ex-*bayanus*). The alcoholic rosé wine obtained with *S. cerevisiae* (ex-*bayanus*) thus had a somewhat fruitier profile and was half a point higher in bitterness. However, it should be noted that the two alcoholic wines produced were very similar to each other with only a few subtle differences.

Making non-alcoholic wine is rather complex, since on eliminating the ethanol (generally by vacuum distillation) the wine's organoleptic profile is completely changed (Castro-Muñoz, 2019) due to the loss of many of its characteristic volatile substances during distillation. However, eliminating the ethanol can over-enhance flavors such as acidity and bitterness. In the non-alcoholic wines, the most desirable organoleptic characteristics (aromatic, alcoholic, yeastiness, fruitiness, sweetness, mustiness and citric flavor) were thus substantially reduced and both non-alcoholic rosé wines showed very similar profiles.

In view of the results obtained, it can be concluded that both rosé wines produced with the probiotic and commercial yeasts show a similar fermentation profile in their kinetic, analytical and organoleptic aspects. These results are in good agreement with previous studies that found that *S. cerevisiae* var. *boulardii* has no negative effect on the aroma of alcoholic drinks such as beer (Capece et al., 2018) and provides acceptable sensory attributes (Senkarcinova et al., 2019).

4. Conclusions

Probiotic alcoholic and non-alcoholic rosé wines made with *S. cerevisiae* var. *boulardii* probiotic yeast showed similar characteristics and sensory attributes than a commercial wine produced with *S. cerevisiae* (ex-*bayanus*). *S. cerevisiae* var. *boulardii* fermented the must without causing notable changes in the aroma or flavor, thus preserving the original properties of the vineyard, a desirable characteristic in yeasts used to produce high-quality wines. Yeast viability studies showed that the probiotic *S. cerevisiae* var. *boulardii* yeast survives the high alcohol content produced during fermentation and vacuum distillation. The probiotic rosé wine stored either at 25±0.5ºC or in a refrigerator (~4ºC) kept its probiotic viability for at least six months. For all these reasons, we firmly believe that *S. cerevisiae* var. *boulardii* can be used successfully for large scale wine production and/or in the production of new fermented products with enhanced health benefits.

Author contributions

Conceptualization: J.C-M and Á.S-A. conceived the idea of this work. Experiments: J.C-M, A.T-M., A.C-V., L.P-C., M.M.; Data curation: A.T-M., Á.S-A. J.M-C., Formal analysis: Á.S-A.; Supervision: Á.S-A., J.M-C. and M.M; Investigation: A.T-M., A.C-V., M.M., J.M-C., M.M and Á.S-A. Writing – original draft: Á.S-A. and J.M-C.; Writing-reviewing and editing: A.T-M., A.C-V., M.M., J.M-C. and Á.S-A.; Funding acquisition: Á.S-A.

Conflict of interests

The authors declare no competing interests.

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