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SHORT COMMUNICATION

# Use of non-*Saccharomyces* yeasts in the *pris de mousse* of Lambrusco. Microbial evolution through alcoholic fermentation and effect on wine volatile profile

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## ABSTRACT

Lambrusco is a sparkling wine largely produced in the north of Italy, especially in the Emilia region. The possible application of two non-*Saccharomyces* yeasts in the secondary bottle fermentation (Champenoise method) was tested in combination with a commercial strain of *S. cerevisiae* to obtain wines having a distinctive volatile profile. Our results demonstrated that the gradual increase of ethanol content in the pied de cuve ensured the adaptation of *Hanseniaspora guilliermondii* and *Torulaspora delbrueckii* at the bottle fermentation, with survival comparable with that of *S. cerevisiae*, in the order of 6 log units. The simultaneous presence of two yeast species reduced the maximum fermentation rate, without any relevant alteration in the main oenological parameters of resultant wines. GC MS-MS analysis of the volatile profile of wines (46 compounds) highlighted differences in wine made from a pure culture of S. cerevisiae from wines obtained by mixed yeasts. Acetates, esters, and fatty acids are the classes of volatile compounds mostly affected by using different yeasts in the bottle fermentation of Lambrusco wines. This work provided for the first time information about the volatile profile of Lambrusco and suggests an innovative application of non-*Saccharomyces* yeast in the production of sparkling wines by champenoise methods.

**KEYWORDS:** Lambrusco sorbara, Lambrusco Marani, Champenoise methods, sparkling wine, *Hanseniaspora guilliermondii*, *Torulaspora delbrueckii*

## INTRODUCTION

Lambrusco wine is produced in Emilia (Italy) by different *V. vinifera* cultivars, the most widespread are *Lambrusco salamino, L. grasparossa, L. Sorbara,* and *L. marani*. Lambrusco is one of the major wine productions in Italy. In 2021, about 170 million bottles were produced, corresponding to 490 million euros in value (Lambrusco - I numeri del vino, 2021). Today, Lambrusco is a red, sparkling, dry or semi-sweet wine, consumed in the early months after the harvest. To obtain this Lambrusco style the winemaking follows the Charmat method, with a one-step alcoholic fermentation in an autoclave and early isobaric bottling. On the contrary, traditionally Lambrusco wines were made with the Champenoise method (Favaro *et al.,* 2017), but in the last decades bottle-fermented Lambrusco almost disappeared because was considered a poor-quality wine. Recently many Lambrusco producers have rediscovered this technique, as it could yield more long-lasting and complex wine, which has found increasing appreciation from consumers. To date, knowledge regarding the Lambrusco wines is scarce, in-depth studies are necessary to improve the quality of Lambrusco made through the Champenoise method.

The Champenoise method is the oldest technique for producing sparkling wines (Buxaderas and Lopez-Tamames, 2012). Yeast finds during bottle-fermentation harsh conditions for its development. The resistance mechanisms activated by yeast in bottle fermentation are partly like that of primary alcoholic fermentation, due to the occurrence of the same limiting factors (ethanol, low pH, nitrogen starvation), but it showed some peculiarity related to environmental bottle conditions (low temperature, $CO_2$, overpressure) (Porras-Agüera *et al.,* 2021; Penacho *et al.*, 2012). Ethanol is the main factor that activates gene transcription during bottle fermentation, inducing the expression of genes involved in respiratory metabolism, in the response to oxidative stress, autophagy, and peroxisomal function (Marks *et al.,* 2008; Rossignol *et al.,* 2003). In addition, low temperature seems to influence the gene transcription profile of yeast during sparkling wine production (García-Ríos *et al.,* 2017).

An accurate adaptation of yeast is essential to stimulate the mechanisms of stress response (Guzzon and Larcher, 2015). Equally fundamental is the supplementation of the wine with the nutritional factors that have been depleted during the first alcoholic fermentation (Costa *et al.,* 2018). If these requirements are ensured, bottle fermentation occurs within a couple of weeks. Subsequently, the ageing of wine begins in contact with the yeast cells which, lysing gradually, release a wide range of chemical fractions: polyphenols, peptides, proteins, amino acids, and lipids (Gnoinski *et al.,* 2021; Moreno Arribas *et al.,* 1996). The release of cell fractions during bottle ageing modifies the organoleptic profile of sparkling wine and guarantees its longevity, thanks to the antioxidant activity of some cell compounds such as gallic acid, *trans*-resveratrol, catechin, and epicatechin, procyanidins and B complex vitamins (Martinez-Garcia *et al.,* 2021; Perez-Magarino *et al.,* 2015; Stefenon *et al.,* 2010).

For their high resistance to ethanol, yeast strains for bottle fermentation are generally chosen from within the *Saccharomyces* genus. However, there is evidence that some non-*Saccharomyces* yeasts are resistant to ethanol concentrations over 10 % (Guzzon *et al.,* 2022; Catrileo *et al.,* 2020; Antoce *et al.,* 2011; Xue *et al.,* 2009). Non-*Saccharomyces* yeasts represent a promising resource in oenology (Di Gianvito *et al.,* 2022; He *et al.,* 2022). Biocontrol, increasing wine mouthfeel by releasing mannoproteins, and improving wine aromatic profile thanks to overexpressed enzymatic activities, are just some of the applications of these microorganisms that are now available on the market as active dry yeasts (OIV, 2017). Among the non-*Saccharomyces* yeasts, two species deserve particular interest considering the scope of this work: *Torulaspora delbrueckii* and *Hanseniaspora uvarum*. *T. delbrueckii* have been involved in fermentation with *S. cerevisiae* in winemaking since the 1990s, when its positive contribution to the aromatic profile of wine (Moreno *et al.,* 1991) and its ability to maintain biological activity at low temperatures were highlighted (Charoenchai *et al.,* 1998). The application of *T. delbrueckii* in sparkling wine production was suggested by Canonico *et al.* (2018). More recently, Silva-Sousa *et al.* (2022) compared the activity of 40 strains of *T. delbrueckii*, confirming the preference of this yeast for low fermentation temperature and noting ethanol resistance up to 18 %. The use of *Hanseniaspora* spp. in winemaking is less widespread, but its presence in the wine environment is ascertained (Mancic *et al.,* 2022). Its enzymatic pathway is broad and of great interest in terms of impact on the volatile profile of wines. An ethanol resistance of up to 12.5 % suggests the possible use of *Hanseniaspora* in sparkling wine production (Moreira *et al.,* 2011).

In this work, *H. uvarum* and *T. delbrueckii* have been used in mixed culture with *S. cerevisiae* as a starter of bottle fermentation of *L. Sorbara* and *L. marani*. The activity of the three yeast species was monitored according to international OIV standards (2010), and the wines obtained were characterised by GC MS-MS analysis to highlight the peculiarities due to the contribution of different yeasts.

## MATERIALS AND METHODS

### 1. Winemaking procedure

*Lambrusco sorbara* was sourced in Sorbara, Italy (44.750, 11.003), while *Lambrusco marani* came from a vineyard located in Montecchio, Italy (44.735, 10.448). Grapes were manually harvested in the first decade of September, and then further transported to the Medici Ermete & Figli winery (Reggio Emilia, Italy). Grapes were pressed (Maximum pressure 0.3 bar, duration 1.5 hours) by a Bucher Vaslin (France) XPert 115 apparatus, without destemming, and juice was separated from pomace within two hours. During grape pressing the oenological tannins Aromax Gall© (AEB, Italy, 2 g/kg) and Protan Malbech© (AEB, 0.12 g/kg) were

**TABLE 1.** Chemical composition of base-wine utilised for sparkling wine production and sparkling wines after bottle-fermentation, mean data (n = 3). We listed only compounds that counted more than 0.01 mg/L in at least one sample. SC: fermentation made by pure *S. cerevisiae*; TD: fermentation made by *S. cerevisiae* + *T. delbrueckii*; HU: fermentation made by *S. cerevisiae* + *H. uvarum*. a, b, c: statistically different in the same wine (One-way ANOVA + Tukey test, p = 0.05).

| | *L. Sorbara* | | | | *L. marani* | | | |
|---|---|---|---|---|---|---|---|---|
| | Base wine | SC | TD | HU | Base wine | SC | TD | HU |
| Main oenological parameters | | | | | | | | |
| Pressure (bar) | - | 7.2$^{a}$ | 7.0$^{a}$ | 7.4$^{a}$ | - | 7.4$^{a}$ | 7.8$^{ab}$ | 7.6$^{a}$ |
| Ethanol (% v/v) | 10.1$^{a}$ | 12.1$^{b}$ | 12.1$^{b}$ | 12.1$^{b}$ | 10.0$^{a}$ | 12.0$^{b}$ | 12.0$^{a}$ | 11.9$^{a}$ |
| Sugars (g/L) | 24.9$^{a}$ | < 1$^{b}$ | < 1$^{b}$ | < 1$^{b}$ | 25.1$^{a}$ | < 1$^{b}$ | < 1$^{b}$ | < 1$^{b}$ |
| pH | 3.04$^{a}$ | 3.07$^{a}$ | 3.08$^{a}$ | 3.08$^{a}$ | 2.96$^{a}$ | 3.0$^{a}$ | 2.99$^{a}$ | 2.99$^{a}$ |
| Total acidity (g/L of tartaric acid) | 7.87$^{a}$ | 7.48$^{b}$ | 7.50$^{b}$ | 7.42$^{b}$ | 9.18$^{a}$ | 8.70$^{b}$ | 8.72$^{b}$ | 8.80$^{b}$ |
| Volatile acidity (g/L of acetic acid) | 0.23$^{a}$ | 0.26$^{a}$ | 0.28$^{a}$ | 0.30$^{a}$ | 0.24$^{a}$ | 0.34$^{b}$ | 0.34$^{b}$ | 0.36$^{b}$ |
| Tartaric acid (g/L) | 2.34$^{a}$ | 2.24$^{a}$ | 2.28$^{a}$ | 2.24$^{a}$ | 2.58$^{a}$ | 2.46$^{a}$ | 2.45$^{a}$ | 2.46$^{a}$ |
| Malic acid (g/L) | 3.46$^{a}$ | 3.48$^{a}$ | 3.42$^{a}$ | 3.37$^{b}$ | 4.06$^{a}$ | 4.09$^{a}$ | 4.06$^{a}$ | 4.01$^{b}$ |
| Volatile compounds (mg/L) | | | | | | | | |
| Isobutytric acid | 0.4524$^{a}$ | 0.8232$^{ab}$ | 1.1020$^{b}$ | 0.8500$^{ab}$ | 0.5415$^{a}$ | 0.8239$^{b}$ | 1.0133$^{c}$ | 0.7970$^{b}$ |
| Butanoic acid | 0.5390$^{a}$ | 0.5539$^{a}$ | 0.6012$^{ab}$ | 0.6545$^{ab}$ | 1.0624$^{a}$ | 1.0711$^{a}$ | 1.0546$^{a}$ | 1.0344$^{a}$ |
| Isovaleric acid | 0.1058a | 0.1584$^{ab}$ | 0.1850$^{b}$ | 0.1834$^{b}$ | 0.1560$^{a}$ | 0.2287$^{b}$ | 0.2192$^{b}$ | 0.2162$^{b}$ |
| Valeric acid | 0.0287$^{a}$ | 0.0229$^{a}$ | 0.0251$^{a}$ | 0.0264$^{a}$ | 0.0280$^{a}$ | 0.0251$^{a}$ | 0.0261$^{a}$ | 0.0270$^{a}$ |
| Hexanoic acid | 2.3601$^{a}$ | 2.2210$^{a}$ | 2.2229$^{a}$ | 2.1565$^{a}$ | 4.1733$^{a}$ | 3.7696$^{b}$ | 3.4323$^{b}$ | 3.5278$^{b}$ |
| Octanoic acid | 2.3901$^{b}$ | 1.7886$^{a}$ | 1.8920$^{a}$ | 1.6368$^{a}$ | 3.3522$^{a}$ | 2.7745$^{b}$ | 2.5647$^{b}$ | 2.5720$^{b}$ |
| Nonanoic acid | 0.0275$^{a}$ | 0.0384$^{a}$ | 0.0276$^{a}$ | 0.0382$^{a}$ | 0.0188$^{a}$ | 0.0357$^{b}$ | 0.0369$^{b}$ | 0.0275$^{ab}$ |
| Decanoic acid | 7.2698$^{a}$ | 10.085$^{a}$ | 8.2627$^{a}$ | 10.114$^{a}$ | 4.3107$^{a}$ | 8.0144$^{b}$ | 8.6865$^{b}$ | 8.0742$^{b}$ |
| Isobutyl acetate | 0.0024$^{a}$ | 0.0021$^{a}$ | 0.0034$^{a}$ | 0.0014$^{a}$ | 0.0038$^{a}$ | 0.0014$^{b}$ | 0.0018$^{b}$ | 0.0014$^{b}$ |
| *n*-butyl acetate | 0.0026$^{a}$ | 0.0015$^{a}$ | 0.0028$^{a}$ | 0.0015$^{a}$ | 0.0039$^{a}$ | 0.0010$^{b}$ | 0.0013$^{b}$ | 0.0015$^{b}$ |
| Isopentyl acetate | 0.1731$^{a}$ | 0.0690$^{a}$ | 0.2869$^{b}$ | 0.0673$^{a}$ | 0.3088$^{a}$ | 0.0520$^{b}$ | 0.0469$^{b}$ | 0.0502$^{b}$ |
| *n*-hexyl acetate | 0.0307$^{a}$ | 0.0334$^{a}$ | 0.0317$^{a}$ | 0.0312$^{a}$ | 0.0445$^{a}$ | 0.0434$^{a}$ | 0.0460$^{a}$ | 0.0480$^{a}$ |
| Ethyl lactate | 5.8356$^{a}$ | 6.8597$^{b}$ | 6.2251$^{b}$ | 6.6535$^{b}$ | 15.6236$^{a}$ | 10.940$^{b}$ | 10.721$^{b}$ | 11.076$^{b}$ |
| Ethyl phenyl acetate | 0.0039$^{a}$ | 0.0052$^{b}$ | 0.0048$^{a}$ | 0.0050$^{b}$ | 0.0011$^{a}$ | 0.0024$^{b}$ | 0.0029$^{b}$ | 0.0030$^{b}$ |
| 2-phenylethyl acetate | 0.0225$^{ab}$ | 0.0108$^{a}$ | 0.0303$^{b}$ | 0.0104$^{a}$ | 0.0275$^{a}$ | 0.0051$^{b}$ | 0.0057$^{b}$ | 0.0051$^{b}$ |
| Ethyl butyrate + ethyl isobutyrate | 0.0933$^{a}$ | 0.1245$^{b}$ | 0.1188$^{b}$ | 0.1210$^{b}$ | 0.1833$^{a}$ | 0.1699$^{a}$ | 0.1936$^{a}$ | 0.1723$^{a}$ |
| Ethyl-2-methylbuthyrate | 0.0050$^{a}$ | 0.0133$^{b}$ | 0.0101$^{b}$ | 0.0132$^{b}$ | 0.0057$^{a}$ | 0.0184$^{b}$ | 0.0165$^{b}$ | 0.0166$^{b}$ |
| Ethyl pentanoate | 0.0010$^{a}$ | 0.0012$^{a}$ | 0.0010$^{a}$ | 0.0011$^{a}$ | 0.0010$^{a}$ | 0.0013$^{a}$ | 0.0010$^{a}$ | 0.0011$^{a}$ |
| Ethyl hexanoate | 0.2921$^{a}$ | 0.3276$^{b}$ | 0.2851$^{a}$ | 0.2675$^{a}$ | 0.4653$^{a}$ | 0.5682$^{a}$ | 0.5012$^{a}$ | 0.4761$^{a}$ |
| Ethyl octanoate | 0.3367$^{a}$ | 0.4593$^{b}$ | 0.4261$^{b}$ | 0.3727$^{ab}$ | 0.4220$^{a}$ | 0.5204$^{b}$ | 0.4266$^{a}$ | 0.4428$^{a}$ |
| Ethyl decanoate | 0.0683$^{a}$ | 0.2505$^{b}$ | 0.1584$^{ab}$ | 0.2158$^{b}$ | 0.0601$^{a}$ | 0.3424$^{a}$ | 0.0685$^{a}$ | 0.1027$^{a}$ |
| Ethyl dodecanoate | 0.0039$^{a}$ | 0.0320$^{b}$ | 0.0085$^{ab}$ | 0.0177$^{ab}$ | 0.0041$^{a}$ | 0.0335$^{a}$ | 0.0041$^{a}$ | 0.0052$^{a}$ |
| Diethyl-succinate | 2.0133$^{a}$ | 4.1797$^{b}$ | 2.5075$^{a}$ | 4.0518$^{b}$ | 1.1585$^{a}$ | 3.4569$^{b}$ | 3.6335$^{b}$ | 3.3720$^{b}$ |
| 1-hexanol | 1.1045$^{a}$ | 1.0312$^{b}$ | 1.0491$^{b}$ | 1.0404$^{b}$ | 0.5144$^{a}$ | 0.4753$^{b}$ | 0.4230$^{b}$ | 0.4371$^{b}$ |
| *trans*-3-hexen-1-ol | 0.0662$^{a}$ | 0.0719$^{a}$ | 0.0677$^{a}$ | 0.0620$^{a}$ | 0.0183$^{a}$ | 0.0150$^{a}$ | 0.0203$^{a}$ | 0.0190$^{a}$ |
| *cis*-3-hexen-1-ol | 0.0652$^{a}$ | 0.0671$^{a}$ | 0.0682$^{a}$ | 0.0708$^{a}$ | 0.0575$^{a}$ | 0.0626$^{a}$ | 0.0572$^{a}$ | 0.0562$^{a}$ |
| Benzyl alcohol | 0.0584$^{a}$ | 0.0570$^{a}$ | 0.0625$^{a}$ | 0.0609$^{a}$ | 0.0508$^{a}$ | 0.0214$^{b}$ | 0.0246$^{b}$ | 0.0262$^{b}$ |
| 2-phenylethanol | 19.0783$^{a}$ | 19.8911$^{a}$ | 18.7320$^{a}$ | 21.0474$^{a}$ | 9.702$^{a}$ | 10.199$^{ab}$ | 11.025$^{ab}$ | 12.1860$^{b}$ |
| Linalol oxide A | 0.0023$^{a}$ | 0.0127$^{b}$ | 0.0109$^{b}$ | 0.0142$^{b}$ | 0.0028$^{a}$ | 0.0138$^{b}$ | 0.0152$^{b}$ | 0.0173$^{b}$ |
| Linalol oxide B | 0.0022$^{a}$ | 0.0088$^{b}$ | 0.0092$^{b}$ | 0.0099$^{b}$ | 0.0011$^{a}$ | 0.0077$^{b}$ | 0.0101$^{b}$ | 0.0099$^{b}$ |
| Linalool | 0.0070$^{a}$ | 0.0190$^{b}$ | 0.0160$^{b}$ | 0.0178$^{b}$ | 0.0101$^{a}$ | 0.0158$^{b}$ | 0.0141$^{b}$ | 0.0151$^{b}$ |
| Alpha-terpineol | 0.0068$^{a}$ | 0.0089$^{b}$ | 0.0080$^{b}$ | 0.0085$^{b}$ | 0.0058$^{a}$ | 0.0057$^{a}$ | 0.0054$^{a}$ | 0.0059$^{a}$ |

added as antioxidant and colour-stabilising substances. Also, the clarifying agent bentonite based Enoclear© (AEB, 0.01 g/kg) was added to improve must clarification before fermentation. Before yeast inoculum, grape must was integrated by Superstart® Blanc (Laffort SA, France, 0.11 g/kg) as a source of organic nitrogen, vitamins, mineral elements, fatty acids, and sterols. Sulphur dioxide was added by utilising the commercial preparation Solfosol-C© (Enartis, Italy, 0.15 g/kg). Alcoholic fermentation was performed in a stainless steel tank of 25 hL of capacity, at 20 ± 2 °C by ADY EC-1118 (Lallemand Inc., Canda, 0.3 g/kg). After alcoholic fermentation, wines were cold stabilised (8 ± 2 °C) for 4 weeks. Each trial consisted of 25 L of base wine, corresponding to 30 bottles of sparkling wine. Prior to bottling the wines (Table 1), sugar (25 g/L) and yeast's nutrient (Nutriferm Arom plus©, Enartis) were added to reach a level of Yeast Assailable Nitrogen (YAN) of 250 mg/L and the pied de cuve containing yeasts prepared as described in the next paragraph in the ratio of 1 % v/v. Bottling was performed using ordinary "champagne" bottles having a nominal volume of 0.75 L, closed with a crown cap (Pe.Di. S.r.l., Italy); fermentation was performed at 20 ± 2 °C, and its evolution was monitored by an AFR-VN01 aphometer (VM meccanica, Italy) installed on the bottle cap (n = 3 for each trial). When the pressure was stabilised, bottles were placed, lying down, in the ageing cell at 15 ± 2 °C for one year.

### 2. Preparation of pied de cuve for bottle fermentation.

The microorganisms involved in the preparation of pied de cuve are *S. cerevisiae* DV10 (Lallemand), *H. guilliermondii* NCYC 2380, and *Torulaspora delbrueckii* BIODIVA TM (Lallemand). Active dry yeasts were rehydrated according to the OIV protocol (2009), while the strain NCYC 2380 was cultured in YM broth for 3 days at 25 ± 2 °C, reaching a concentration of $10^8$ CFU/mL. Each yeast strain was multiplied in a 3:1 water:wine mixture, containing 100 g/L of sucrose (Sigma Aldrich, MO) and 0.5 g/L of Nutriferm Arom Plus© (Enartis). The volume of each yeast culture was 1 L, and they were incubated for 24 h at 25 ± 2 °C, under agitation. After this first step, each yeast culture was increased in volume adding, every 12 hours, a mixture of wine/water and sucrose in the ratio 25:65:10 %. The addition was repeated 3 times until a volume of 2.5 litres was reached. The wine/water ratio was regulated at 25:75 (1st step), 50:50 (2nd step) and 75:25 (3rd step). At the end of multiplication, the pied de cuve was added to the wine in a ratio of 1 % of the total base wine volume.

### 3. Microbiological analysis

The preparation of pied de cuve and bottle fermentation was monitored by plate count (WL Agar medium for total yeast and Lysine Agar medium for non-*Saccharomyces* yeast, both purchased by Oxoid, UK) and microscopical yeast count, according to the OIV methods (2010). The attribution of species to the non-*Saccharomyces* yeast growth onto the plate was confirmed by specie-specific PCR, as proposed by van Breda *et al.* (2013) and Santiago-Urbina *et al.* (2015) on a representative number of colonies grown onto WL/Lysine Agar.

### 4. Chemical analysis

Basic chemical analysis of wines (Sugars, pH, ethanol, total acidity, acetic and malic acids, yeast assailable nitrogen, and total/free $SO_2$) was performed by a WineScan $SO_2$ FT-IR analyser (FOSS, DK). The analysis of volatile compounds was performed according to the method proposed by Paolini *et al.* (2018), while volatile compounds were sampled by solid-phase extraction (SPE) using ENV+ cartridges. The GC-MS/MS analysis was performed by an Agilent (CA) Intuvo 9000 apparatus, coupled with an Agilent 7000 Series Triple Quadrupole mass spectrometer working in electron impact mode at 70 eV. The mass spectrum was acquired in Multiple Reaction Monitoring mode, setting the instrument in a dynamic system.

### 5. Statistics

ANOVA One-way, Partial Least Squares regression (PLS), and Principal Component Analysis (PCA) on the set of data were performed by TIBCO Statistica® 14.1.0 (TIBCO Software, CA).

## RESULTS

### 1. Evolution of yeast during pied de cuve preparation and bottle fermentation

Table 2 shows the evolution of the four pied de cuve produced by different yeasts in terms of cell density and sugars/ethanol concentration. The pied de cuve produced by pure *S. cerevisiae* reached a cell concentration of $1.5 \times 10^8$ CFU/mL in 48 hours. In the pied de cuve made by mixed yeast culture, the concentration of each strain was initially lower because the mixing of strains was performed immediately prior to the bottling, to avoid competition between the two species. *H. uvarum* and *T. delbrueckii* reached a cellular concentration over the 7 log units (Table 2). *T. delbrueckii* appeared more able to accumulate ethanol, and the 10.2 % v/v was reached after 48 hours with a cellular concentration of $7.7 \times 10^7$ CFU/mL. *H. uvarum* reached $8.5 \times 10^7$ CFU/mL with an ethanol content of 9.5 % v/v. At bottling time, the total yeast concentration was $1.3 \pm 0.5 \times 10^6$ CFU/mL in the test performed by pure *S. cerevisiae,* $2.2 \pm 0.6 \times 10^6$ CFU/mL in the tests performed by *S. cerevisiae* and *T. delbrueckii*, and $2.1 \pm 0.2 \times 10^6$ CFU/mL in the test performed by *S. cerevisiae* and *H. uvarum*. For both mixed cultures, the ratio between *Saccharomyces* and non-*Saccharomyces* was adjusted to 1:1.

Monitoring yeast viability continued during bottle fermentation. In Figure 1, the concentration of *S. cerevisiae, T. delbrueckii*, and *H. uvarum* was represented at 3, 8, and 12 days after wine bottling, as the mean of both tests performed on *L. Sorbara* and *L. marani* wines (n = 6), because the differences observed between the two varieties were non-statistically significant (One-way ANOVA, $p > 0.05$). At the beginning of bottle fermentation in the test by pure *S. cerevisiae*, the total concentration of yeast was $4.4 \pm 0.1 \times 10^6$ CFU/mL with a concentration of

**TABLE 2.** Evolution of chemical and microbiological parameters of pied de cuve employed in bottle fermentation (n = 1).

| Pied de cuve | Sampling time | Yeast | Ethanol | Sugars |
|---|---|---|---|---|
| | h | $\times 10^7$ CFU/mL | % v/v | g/L |
| Pure *S. cerevisiae* | 8 | 9.8 | 5.5 | 93.4 |
| | 24 | 12.5 | 9.8 | 51.5 |
| | 48 | 15.1 | 10.8 | 33.4 |
| *S. cerevisiae* 50 % | 8 | 5.7 | 5.0 | 95.2 |
| | 24 | 7.9 | 9.4 | 71.2 |
| | 48 | 7.6 | 10.6 | 44.1 |
| *H. uvarum* | 8 | 5.0 | 3.8 | 94.6 |
| | 24 | 7.7 | 8.2 | 53.5 |
| | 48 | 8.5 | 9.5 | 30.6 |
| *T. delbrueckii* | 8 | 6.1 | 5.2 | 94.9 |
| | 24 | 6.8 | 9.6 | 68.6 |
| | 48 | 7.7 | 10.2 | 46.8 |

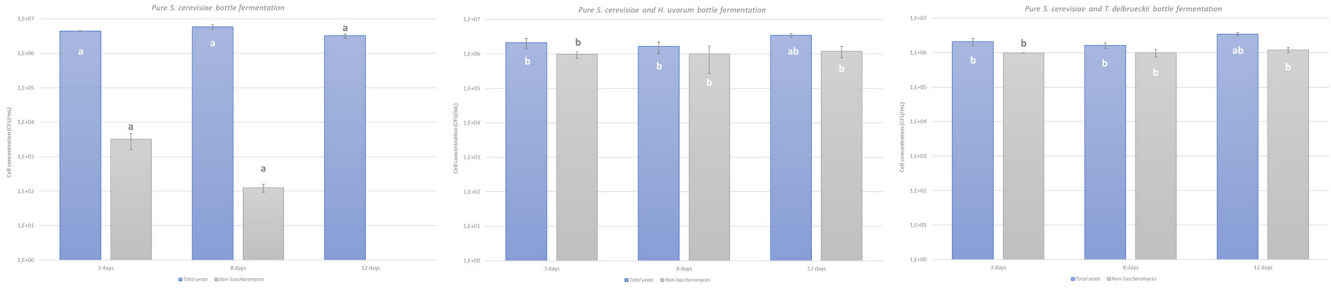


**FIGURE 1.** Yeast concentration measured on days 3, 8 and 12 after wine bottling. Data is expressed as mean ± SD of tests performed by *L. Sorbara* and *L. marani* wines (n = 6) and both *S. cerevisiae* and non-*Saccharomyces* yeasts. [a, b, c]: statistically different for the same parameter and day in different tests. (One-way Anova + Tukey test, p = 0.05).

non-*Saccharomyces* below the 4 log units. The concertation of yeast increased on the 8th day ($5.8 \pm 0.9 \times 10^6$ CFU /mL), and after that, a reduction in cell concentration was observed ($3.2 \pm 0.4 \times 10^6$ CFU /mL), probably due to the exhaustion of sugars. In the test performed by *S. cerevisiae* and *H. uvarum*, the total yeast concentration remained at the interval between $2.1 \pm 0.7$ and $3.4 \pm 0.4 \times 10^6$ CFU/mL and $9.8 \pm 2.3 \times 10^5$ and $1.2 \pm 0.4 \times 10^6$ CFU/mL for *H. uvarum*. Worth noting was the concentration of *H. uvarum,* over the 6 log units after 12 days of fermentation, in the presence of high ethanol content, in the harsh environment of sparkling wine. As regards fermentation by *S. cerevisiae* and *T. delbrueckii*, the non-*Saccharomyces* yeast started from the lowest concentration ($3.9 \pm 0.1 \times 10^5$ CFU/mL) but settled between the 5 and 6 log units for the entire duration of bottle fermentation. At the beginning of the test, the total yeast has a concentration of $2.2 \pm 0.1 \times 10^6$ CFU/mL, reaching the maximum after 12 days of fermentation. The statistical treatment of data revealed significant differences (One-way ANOVA, $p < 0.05$) in the concentration of *S. cerevisiae* in the mixed fermentation, compared to the tests performed by pure yeast culture on the 3rd and 8th days.

## 2. Dynamics of bottle alcoholic fermentation and chemical features of wines

Figure 2A represents the evolution of alcoholic fermentation, with the data expressed as the mean between tests performed on *L. Sorbara* and *L. marani* (n = 6, 3 for each wine). In the test performed by pure *S. cerevisiae*, the alcoholic fermentation started quickly, and the residual sugar was about 15 g/L (2nd day) and 10 g/L (5th day). In the tests performed by mixed yeast culture, the sugar degradation followed a linear trend, with a delay compared to the previous test in the first two points of observation. After that, the fermentation rate increased and the evolution of alcoholic fermentation in the 3 trials proved comparable. In both cases, sugar degradation finished in 20 days. The maximum rate of fermentation ($V_{max}$) was reached on the 2nd day of fermentation (Figure 2B), corresponding to 6.15 ± 0.22 g/L/day for the test performed by pure *S. cerevisiae*, while in the tests performed by mixed yeast culture, the $V_{max}$ was 2.95 ± 0.48 g/L/day in the case of *H. uvarum* + *S. cerevisiae* and 2.50 ± 0.37 g/L/day in the case of *T. delbrueckii* + *S. cerevisiae*. The statistical analysis revealed significant differences (One-way ANOVA, $p < 0.05$) in the number of sugars degraded and in the fermentation

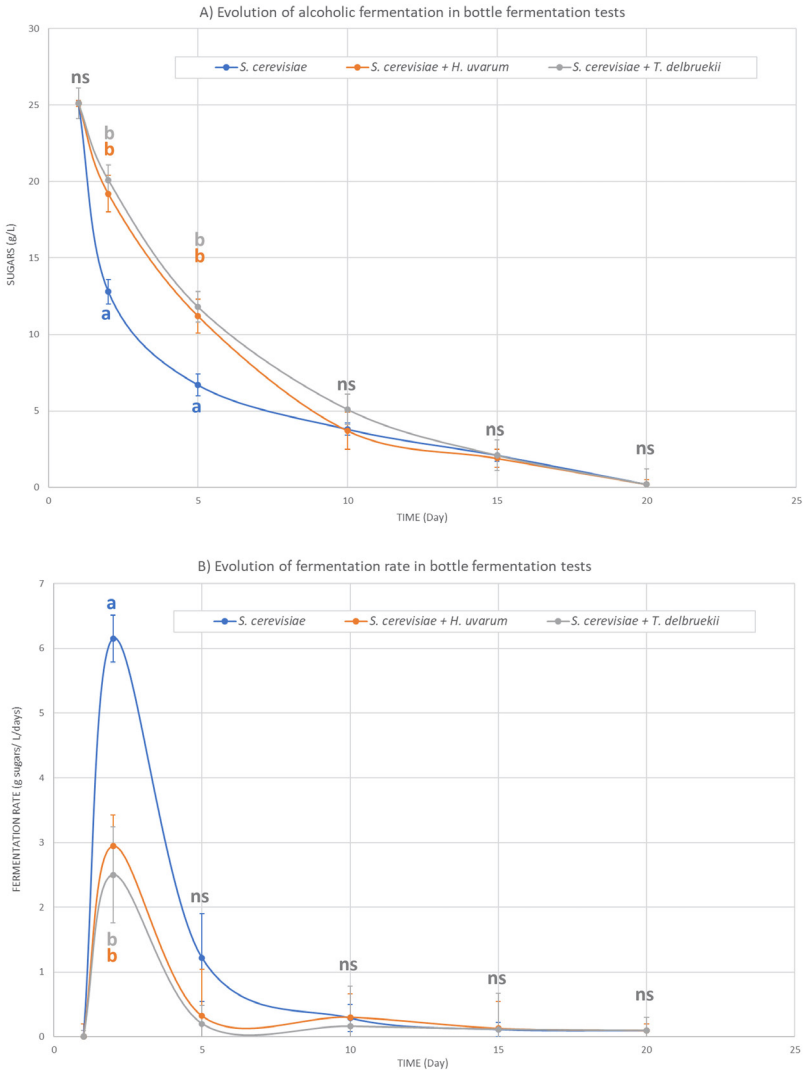


**FIGURE 2.** Evolution of alcoholic fermentation as sugar consumption (A) and fermentation rate (B). Data is expressed as mean ± SD (n=6) of tests performed on *L. Sorbara* and *L. marani* wines. [a, b] statistically different in the same wine (One-way Anova + Tukey test, p = 0.05), ns: differences are not statistically significant (One-way Anova + Tukey test, p = 0.05).

rate until the 10th day of fermentation, comparing pure fermentation and mixed fermentation.

The volatile concentrations are shown in Table 1. Thirty-two aromatic compounds belonging to different chemical classes were identified and quantified. Due to the absence of a large number of aromatic precursors in the Lambrusco grapes, the majority of volatile molecules identified have a fermentative origin, and only seven of them are varietal compounds. Among these were identified the monoterpenes linalool, linalool oxides, and alpha-terpineol. These compounds are directly involved in the floral bouquet, but in the Lambrusco wines their concentration was lower the odour threshold: 0.05 mg/L for linalool, 6 mg/L for linalool oxides, and 0.4 mg/L for alpha-terpineol (Fariña *et al*., 2015). Hexanol, cis-3-hexen-1-ol, and trans-3-hexen-1-ol are the C6-molecules formed during the pre-fermentative stage of winemaking. 1-hexanol in alcoholic beverages imparts green and floral notes, with a sensory threshold of about 1 mg/L (Berger, 2007). As reported in Table 1, only the L. Sorbara wines have a concentration of 1-hexanol that exceeds slightly the threshold limit. Cis-3-hexen-1-ol and trans-3-hexen-1-ol generate unpleasant herbaceous off-odours but they usually occur in low levels and do not contribute to wine aroma. In both the Lambrusco varieties, the concentration of cis-3-hexen-1-ol and trans-3-hexen-1-ol was under the threshold value of 0.4 and 1 mg/L, respectively (Fariña *et al.,* 2015).

2-phenylethanol and benzyl alcohol are derived from grapes through the metabolism of yeast during the fermentation process. 2-phenylethanol is generally a positive contributor to wine aroma, characterised by a pleasant rose-like aroma. *L. Sorbara* shows a high content of 2-phenylethanol above its sensory threshold (10 mg/L) (Fariña *et al*., 2015).

The organic compounds originated from the alcoholic fermentation and quantified in the wine samples were acids, acetates, and ethyl esters. As shown in Table 1, a total of eight acids were identified. Hexanoic acid, octanoic acid, and decanoic acid showed the highest concentration exceeding their odour threshold (0.4, 0.5, and 1 mg/L, respectively) (Fariña *et al*., 2015). At high concentrations (20 mg/L), these compounds have been associated with an unpleasant scent, but low amounts contribute positively to increasing the complexity of the wine aroma. Among the acetates and ethyl esters characterised by fruity descriptors, isopentyl acetate, ethyl hexanoate, ethyl octanoate, diethyl-succinate, and ethyl lactate were the major compounds. Isopentyl acetate and ethyl hexanoate were the only ones that showed a concentration that exceeded their odour threshold (0.03 and 0.014 mg/L, respectively) for all samples, while the odour threshold of ethyl octanoate (0.5 mg/L) was exceeded in only one (Fariña *et al*., 2015).

The statistical treatment of data in Table 1 by different approaches facilitates the compression of differences among wines due to the yeast's activity. ANOVA and Tukey tests found significant differences in volatile compounds between base wines and wines after secondary fermentation. In some cases, we also found differences among the 3 different experimental hypotheses and between wines made by SC with respect to those made by non-*Saccharomyces* yeasts. PCA analysis considered the entire set of compounds. Figure 4 represents the distribution of cases on the plan designed by the first two variables that explained 99.4 % of the entire variability in *L. Sorbara* samples and 93.9 % in *L. marani*. Wines before bottle fermentation proved to be separated from other samples, with negative coordinates from 1$^{st}$ and 2$^{nd}$ variables. This result was linked to the lesser content in all classes of volatile compounds, except for esters in *L. marani* wines. Lambrusco samples made from bottle fermentation by pure *S. cerevisiae* were characterised by a high content of acetates, while wines made from mixed yeast cultures are clearly distinguishable by the higher content of fatty acids and alcohol. The results seem to confirm that the wine volatile profile after bottle fermentation can be modulated by using different yeasts and they will be discussed in the next chapter.

## DISCUSSION

The production of sparkling wines using the Champenoise method is complex and expensive, justified by the greater value that these wines have for consumers than wine produced through the Charmat method. This comparison acquires even greater importance in the case of Lambrusco, which is generally positioned on the market in a medium-to-low range of price. The use of non-*Saccharomyces* yeasts to obtain wines having peculiar organoleptic features has now been widely tested. Promising applications involved "difficult" wines, where the activity of *S. cerevisiae* was partially compromised. In this sense, worthy experiences were conducted on wines made from dried grapes where osmotic stress could alter the response of *S. cerevisiae* (Azzolini *et al.,* 2012). Other interesting studies concerned the enhancement of wines made from autochthonous grape varieties (Arslan *et al.,* 2018; Zhang *et al.,* 2018), or the correction of deficiencies in grape musts, exploiting alternative ways of consuming carbohydrates, as in the case of *L. thermotolerans* (Morata *et al*., 2018). Generally, the activity of non-*Saccharomyces* yeasts is limited to the first days of alcoholic fermentation, because it was believed that they did not tolerate high doses of ethanol or, worse still, could alter wines. One of the main points of interest of this work is the use of non-*Saccharomyces* yeasts for the entire duration of bottle alcoholic fermentation, based on the assumption that an adequate adaptation of yeasts could activate their resistance to limiting factors, such as ethanol, ensuring their prolonged survival in wine. This hypothesis is based on several reports (Yoshida *et al*., 2021; Ma and Liu, 2010), which demonstrate that resistance mechanisms of yeasts are induced by cell growth in the presence of stress factors at the sub-lethal level. In our experience, the progressive increase of ethanol content in pied de cuve did not counteract yeast growth, which reached 7 log units/mL in the presence of 10 % ethanol. *T. delbrueckii* and *H. uvarum* proved capable of surviving in wine, during bottle fermentation, for at least 12 days, maintaining a cellular concentration between 5 and 6 log units/mL. The lack of dominance of *S. cerevisiae*, which in mixed fermentation reached a concentration lower than that of the test by pure strain, suggests a possible competition among different yeast species, as reviewed by Di Gianvito *et al.* (2022).

The evolution of bottle fermentations agreed with the hypothesis of an interaction between different yeasts in mixed cultures, even if all wines regularly concluded the consumption of sugars and no alterations of the main oenological parameters (acidity, acetic acid, pressure, and alcohol) are observed. Mixed fermentations show an initial delay, compared to fermentations performed by pure *S. cerevisiae,* and a lower $V_{max}$. Further studies are necessary to understand what actions should be taken to optimise yeast performance in mixed fermentations. To our knowledge, this work is the first time that the volatile profiles of wines made from Lambrusco grapes are analysed with advanced mass spectrometry techniques. We have no prior scientific references to use as a comparative basis, and we must, therefore, rely on tasting notes, technical publications (Favaro *et al*., 2017), and the product specification of the main Lambrusco DOC (https://lambrusco.net) to draw some confirmations about the aromatic profiles of the base wines. *L. Sorbara* is generally recognised as a wine characterised by a delicately floral aroma. The high content of 2-phenylethanol would seem to confirm this description (Cordente *et al.,* 2021). On the other hand, *L. marani* is known not to have a profile distinguishable from other varieties of Lambrusco, so much so that it is often used in blends. The high concentration of medium-chain fatty acids and esters suggests an aromatic profile based on fruity notes (Cameleyre *et al.,* 2015). Bottle fermentation by ordinary *S. cerevisiae* enhances the contribution of acetates in the wine profile (Martínez-García *et al.,* 2017; Verzeletti *et al*., 2016), while the presence of non-*Saccharomyces* yeast has

resulted in wines characterised by floral and spicy aromas, typical of higher alcohols and fatty acids, if present in small quantities (Carpena *et al*., 2020).

In conclusion, bottle fermentation influences the aromatic profile of Lambrusco wines, without depletion of molecules that could be related to the distinctive traits of each grape variety. The wine obtained with a pure culture of *S. cerevisiae* appeared to be a transition point, in terms of organoleptic profile, between the base wine and the bottle-fermented Lambrusco obtained by mixed yeast cultures. Further studies will be necessary to establish whether these variations in the aromatic profile are due to an effective contribution of non-*Saccharomyces* yeasts or the altered behaviour of *Saccharomyces*, due to a competition between different species, which can be hypothesised considering the synthetic parameters of the fermentations. A key role can be played by the nutritional integration of the base wine, calibrated on the characteristics of each species, as well as a refinement of the inoculum ratio of the different yeast species (Gallo *et al.,* 2023).

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