

Co-Inoculation of three species

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The adoption of multi-species starter cultures is highly considered in modern winemaking to enhance the complexity and wine attributes. However, the valuation of strains compatibility at the industrial-scale is essential to guarantee the quality and the safety during the process. This concept is particularly important when the starter is composed of non-*Saccharomyces*, *Saccharomyces* spp. and malolactic bacteria, three organisms with dissimilar biological properties and oenological implication.

Keywords: wine ; mixed starter cultures ; *Lactobacillus plantarum* ; *Saccharomyces cerevisiae* ; *Candida zemplinina* ; *Starmerella bacillaris* ; autochthonous yeast ; alcoholic fermentation ; malolactic fermentation

1. Description

The natural ^[1] transformation of red grape must into wine is a complex process driven by the subsequent growth of oenological yeasts and malolactic bacteria ^[2]. The first phase, alcoholic fermentation (AF), is started by several species of non-*Saccharomyces* yeasts (such as *Candida* spp., *Hanseniaspora* spp., and *Pichia* spp.) and driven by strains belonging to *Saccharomyces cerevisiae* species, that complete the fermentation process ^[3]. The effect of the non-*Saccharomyces* species during the AF is significant since they contributed in defining the composition of wine aroma ^[4]. Several non-*Saccharomyces* yeasts, commercialized as oenological starter cultures, have been suggested for use in mixture with *S. cerevisiae* strains ^[5]. The malolactic fermentation (MLF) is a secondary fermentation process that naturally occurs at the end of the AF. *Oenococcus oeni* is the lactic acid bacterium (LAB) principally responsible for this process ^[6], even if several *Lactobacillus plantarum* strains demonstrated to be suitable as MLF starter cultures ^[7].

Candida zemplinina (syn. *Starmerella bacillaris*) is a non-*Saccharomyces* yeast with fructophilic nature and low sugar/ethanol conversion rate ^[8]. The enological significance of *C. zemplinina* strains used in mixture with *S. cerevisiae* has been established ^[9]. In the course of a recent investigation, we have isolated the *C. zemplinina* 35NC1 strain that was able to improve the aromatic complexity of the produced wine ^[10].

The interactions between *S. cerevisiae* ^[11] and non-*Saccharomyces* ^[12] strains with different LAB, such as *L. plantarum* ^[7] ^[1], have been recently investigated, but none of these studies have established the relation among strains belonging to these three categories during a vinification at the industrial scale. In order to cover this lack of knowledge, we used for the first time, three Apulian autochthonous characterized strains belonging to the species *S. cerevisiae* ^[13], *C. zemplinina* ^[10] and *L. plantarum*, as a mixed formulation to inoculate lab-, pilot- and scale large-scale vinification tests (Figure 1).

Figure 1

As first step, four different vinifications of Negroamaro sterilized must were set up at the lab-scale. The Trial 1 was inoculated, as control, with *S. cerevisiae* NP103 strain alone. The second vinification, denoted as Trial 2, was inoculated with the NP103 strain together with *C. zemplinina* 35NC1. In Trial 3 the *S. cerevisiae* strain was co-inoculated with *L.*

plantarum LP44 strain. Finally, the fourth vinification (Trial 4) was inoculated by the addition of all the three above mentioned starter strains, simultaneously. All the vinifications completed the alcoholic fermentation process and, as expected, the wine produced inoculating *L. plantarum* was denoted by the conversion of malic acid into lactic acid. The four wines were then subjected to Gas Chromatographic-Mass Spectrometry (GC-MS): the analysis allowed the identification of 32 different volatile organic compounds (VOCs) belonging to five different families, i.e., alcohols, esters, acids, terpenes and lactones. The Odor Activity Value (OAV) of each volatile compound identified was determined, indicating the potential aroma contribution of individual molecules denoted by $OAV > 1$ [14]. The fruity, floral, herbaceous, fatty, and vinous odorant series were obtained by grouping volatile aroma compounds having $OAV > 1$ and characterized by similar descriptors [15]. The fruity and floral series showed significant differences among the wines, in particular, higher values of OAV are associated with wine fermented by non-*Saccharomyces*, *Saccharomyces*, and *Lactobacillus* as result of high concentrations of esters and linalool. This representation suggested that the complexity of the wine can reflect the complexity of the starter cultures [13][16][17]. To evaluate the oenological and fermentative performances of the newly defined mixed starter formulation, the inoculation scheme tested at lab-scale was validated by carrying out vinifications at pilot-scale, where both the alcoholic and malolactic fermentation took place, as expected.

The GC-MS analysis of volatile compounds in the wines produced by the pilot-scale vinifications allowed the identification of 27 molecules belonging to different chemical classes such as alcohols, esters, volatile acids, terpenes, sulphuric compounds, and volatile phenols. The results of the above assay confirmed that positive influence of both *C. zemplinina* and *L. plantarum* on the aroma of the obtained wines.

Two industrial-scale vinifications were carried out in an industrial winery cellar of Salento (Apulia, Southern Italy) during the vintage 2017 and 2018 by fermenting 10.000 L of Negroamaro grape must, together with control trials, where commercial starters were used. The fermentation processes inoculated with the mixed starter formulation regularly completed and no significant differences among the samples were not found concerning ethanol, total and volatile acidity. The concentrations of volatile molecules were quantified in the wines produced during the two vintages, and they were grouped by chemical class. The results showed that the wines produced with the novel mixed starter had higher concentrations of volatile compounds, such as alcohols, esters and terpenes, molecules that give the highest contribution to the overall wine aroma.

2. Conclusion

Considering the relevance of evaluating new mixed starter cultures at different scales, we further provided a comparative analysis of the mixed starter VOCs production among the different volumes of vinification explored in this study. The statistical analysis of data indicated a clear separation among lab-scale, pilot-scale, and industrial-scale trials, suggesting that VOCs differently contribute to the chemical diversity of produced wines. These findings underlined the importance to explore the oenological and biological consequences of a different winemaking scales in the evaluation of the impact of new starter cultures on wine aroma.

To the best of our knowledge, this work firstly tested the utilization of a mixed starter culture including species belonging to *C. zemplinina*, *S. cerevisiae*, and *L. plantarum* for production of wine at the industrial scale. The results underlined the modulation of volatiles profile due to the different combinations of microbial strains used, with particular attention to the practice of simultaneous inoculation. Finally, this work highlights the relevance of a full-scale assessment to define the contribution of a multi-species starter culture in terms of VOCs diversity.

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