**Microbial interactions during fermentations in winemaking**

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The early disappearance of non-*Saccharomyces* yeasts in mixed alcoholic fermentations for wine production has not yet been fully explained by classical microbiology. Applying RNA sequencing on yeasts’ cells obtained by fermentation in real red grape must should provide insights to the microbial responses occurring in mixed fermentations. Fermentations with *Starmerella bacillaris* and *Saccharomyces cerevisiae* were performed in Nebbiolo must using different conditions (pure, mixed, and mixed with physical separation of cells). Microbiological and chemical analyses data was in line with previous studies. Cells were collected and frozen stored and RNA is going to be extracted with the protocol that has been set up the previous year and sequenced.

**Interazioni microbiche durante la fermentazione in vinificazione**

La rapida scomparsa dei lieviti non-*Saccharomyces* nella fermentazione alcolica per la produzione del vino non è stata completamente spiegata dalla microbiologia classica. L’applicazione di RNA sequencing sulle cellule di lievito ottenute da fermentazioni in mosto d’uva rossa naturale potrebbe fornire alcune risposte alle modifiche di espressione genica che avvengono durante la fermentazione. Fermentazioni con *Starmerella bacillaris* e *Saccharomyces cerevisiae* sono state svolte in mosto Nebbiolo usando diverse condizioni (in purezza, mista e mista con separazione fisica delle cellule). I risultati delle analisi chimiche e microbiologiche sono in linea con lavori precedenti. Le cellule sono state raccolte, stoccate surgelate e l’RNA verrà estratto con il protocollo messo a punto l’anno scorso per il successivo sequenziamento.

1. Introduction

Many studies demonstrated that the evolution of the must from microbiological and chemical point of view during the fermentation is one of the causes that lead to the early disappearance of non-*Saccharomyces* yeast. It was demonstrated that the concentration of ethanol or other toxic compounds, as the depletion of nutrients are not the unique cause of this behavior (Niessen et al., 2003). Englezos et al. (2019) studied the consequences of the physical contact of *Saccharomyces cerevisiae* and *Starmerella bacillaris* cells in growth dynamics modulation. In case of cells’ separation, the non-*Saccharomyces* yeast was able to stay alive longer than in case of contact. But, due to the inability of the classical microbiological tools to investigate the origin of these findings, a transcriptomics approach to study the changes correlated to the physical contact of cells was proposed. RNA sequencing provides insights into the expression of genes, indicating which metabolic pathways are over- or under expressed. To the best of our knowledge, there is a lack of information regarding the study of yeast-yeast interactions involved in mixed fermentation in a real red grape must.

2. Materials and Methods

To simulate the real red wine fermentation conditions, a natural must of Nebbiolo grapes was used. Must was pasteurized at 70°C for 2 hours to ensure the absence of viable microorganisms (confirmed by spreading on WLN medium). Three 500 mL flasks with 200 mL of must and the two compartments of the bioreactor (200 mL each one) separated by a filtering membrane (0.45 μm), were used in this study. All trials were performed in triplicate. The yeasts used were a commercial *S. cerevisiae* (Uvaferm BC, Lallemand, Verona) and two different strains of *Starm. bacillaris* (FC54 and MUT5705); their inoculation concentration was 6.0 Log/mL. The mixed fermentations were performed applying a sequential inoculum of the *S. cerevisiae* after 48 hours. Pure fermentations with each yeast were carried out as control together with two mixed with and without physical contact. Fermentations were conducted at 25°C for 14 days. Samples for RNA extraction were collected at day 2 for pure *S. cerevisiae*, at day 4 for pure *Starm. bacillaris*, at day 2 plus 4 hours for the mixed fermentations, and at day 7 for all; the delay for the pure fermentations has the goal to collect cells from similar must’s conditions. Sampling for microbiological and chemical analysis were done at day 0, 2 (for pure fermentations), 2 plus 4 hours (for mixed fermentations), 4, 7, 10, and 14. The RNA extraction will be done using the RNeasy Micro Kit (QIAGEN, Milan). The protocol was optimized during the past year applying some modifications to those proposed by the company.

3. Results

The reported results are those collected until the submission of this manuscript. In pure fermentations, both yeasts overcame 8 Log/mL (at day 2 for *S. cerevisiae* Uvaferm BC and 4 for *Starm. bacillaris* FC54) and then begun to decline, more quickly in case of *S. cerevisiae*. Cells’ concentration of *Starm. bacillaris* was higher in both mixed fermentations than in case of pure. In this environment, *S. cerevisiae* was not able to reach the same high concentration as in pure culture. In case of physical contact, the highest concentration was found at day 7 and was lower than 7.5 Log/mL. Plate counts confirmed the quicker disappearance of the non-*Saccharomyces* yeast when involved in mixed fermentation with *S. cerevisiae* compared to the case of physical separation. Differently from previous work (Englezos et al., 2019), *Starm. bacillaris* was not found (<10 CFU/mL) in mixed fermentation in bioreactor at the end point (Table 1). In this case, it was absent at the 14th day while in fermentation conducted in flask it was at the 10th.

**Table 1** Cells count on WLN medium. Values expressed as Log/mL

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Pure | Pure | Mixed flask | Mixed bioreactor |
| Sampling day | *Starm. bacillaris* | *S. cerevisiae* | *Starm. bacillaris* | *S. cerevisiae* | *Starm. bacillaris* | *S. cerevisiae* |
| D0 | 6.1 ± 0.2 | 5.9 ± 0.1 | 6.1 ± 0.2 |   | 6.1 ± 0.2 |   |
| D2D2 + 4 h | 7.9 ± 0.1 | 8.1 ± 0.0 | 8.0 ± 0.1 | 6.2 ± 0.4 | 8.3 ± 0.2 | 5.9 ± 0.0 |
| D4 | 8.1 ± 0.0 | 7.8 ± 0.1 | 8.2 ± 0.1 | 7.2 ± 0.1 | 8.4 ± 0.2 | 7.9 ± 0.1 |
| D7 | 8.0 ± 0.0 | 7.7 ± 0.0 | 7.7 ± 0.1 | 7.4 ± 0.1 | 8.5 ± 0.4 | 7.7 ± 0.2 |
| D10 | 7.9 ± 0.1 | 7.1 ± 0.3 | <1 | 7.2 ± 0.2 | 5.3 ± 1.3 | 7.4 ± 0.5 |
| D14 | 7.1 ± 0.3 | 5.8 ± 0.5 | <1 | 7.1 ± 0.2 | <1 | 7.0 ± 0.3 |

The Nebbiolo grape must used had a concentration of total sugars of 246.39 g/L (glucose 120.37 g/L, fructose 126.02 g/L). *Starm. bacillaris* was not able to ferment all the available sugars (residue of 70.86 g/L mainly represented by glucose) and produced a final amount of ethanol of 11.36 % (v/v). *S. cerevisiae* and the mixed fermentation in flask went dry and produced high amount of ethanol (15.82 % (v/v) and 15.68 % (v/v), respectively), while this didn’t happen in mixed fermentation conducted in bioreactor where the highest amount of glycerol (around 10.53 g/L) was produced and ethanol was reduced by about 1 % (v/v), if compared to the control fermented by *S. cerevisiae* in pureness.

4. Discussion

With the goal to study the interactions occurring between a non-*Saccharomyces* yeast and *S. cerevisiae*, transcriptomic analysis is going to be applied on the RNA extracted from the cells collected under different fermentation conditions. The choice of two different days for the first sampling point of cells collection for pure fermentations to have similar conditions of the must was confirmed by HPLC (sugars and ethanol nearly equal; data not shown). The quicker disappearance of *S. cerevisiae* in pure fermentation probably is due to the complete depletion of nutrients. The highest amount of *Starm. bacillaris* cells in mixed fermentation should be correlated to a response due to the competition with another yeast. While *S. cerevisiae* overpassed 8 Log/mL at the second day in pure fermentation, this was not achieved in case of mixed; this should be correlated to factors that reduce its ability to reach high cell concentration. This behavior is more evident in case of mixed fermentation with contact, hypothesizing a disturbance due to the presence of high concentration of a competing yeast (for space and nutrients). As was studied in the past, the physical separation of cells allows a longer permanence of the non-*Saccharomyces* yeast. In this last case, the presence of *Starm. bacillaris* lasted until the tenth day while it was undetectable in case of fermentations with contact. Viable cells of *Starm. bacillaris* was not found at the fourteenth day in mixed fermentation with physical separation. The ethanol concentration (14.97 % (v/v)), that is higher than previous studies done on this couple of yeasts (Englezos et al., 2016), may have played a role in its earlier suppression. The longer presence of *Starm. bacillaris* lowered the ethanol and increased the glycerol (10.53 g/L) as well. It is expected that the transcriptome analysis will lead to a better understanding of the phenomena observed during mixed fermentations.

5. References

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