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Impact of sequential inoculation with *M. pulcherrima* on wine properties and influence of the main characteristics of the must

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Over the past 50 years, the use of selected *S. cerevisiae* starters to control alcoholic fermentation has broadened, to ensure the completion of fermentation and to avoid the production of undesirable off-compounds. Non-*Saccharomyces* yeasts, naturally predominant in grape must, are rapidly outcompeted. Although these species were long viewed as spoilage yeasts, their potential for improving the sensory quality of wines is now acknowledged. They possess some specific metabolism pathways and excrete hydrolytic enzymes involved in the release of varietal aromas. Since their use is recent, their behavior during fermentation, in particular the interactions with *S. cerevisiae* in mixed culture, remain little known.

The contribution of *M. pulcherrima* in sequential inoculation with *S. cerevisiae* to wine fermentation was investigated in this study. With this aim, we analyzed the impact of the main components of grape, i.e. concentrations of sugars, nitrogen and lipids, on the production of aroma compounds (higher alcohols, acetate and ethyl esters and thiols) during *M. pulcherrima* / *S. cerevisiae* sequential fermentation.

Materials and Methods

Experimental design

- 3 concentrations of :
- **sugar** (glucose + fructose),
 - **lipids** (phytosterols)
 - **nitrogen** (ammonium + amino acids)

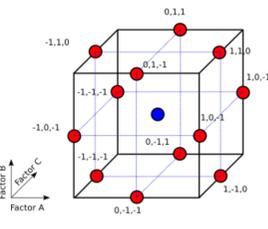
Variables Levels	Sugar (g/L)	Nitrogen (mgN/L)	Lipids (mg/L)
-1	180	80	2
0	220	190	5
+1	260	300	8

Box-Behnken design:

- 15 fermentations with central point in triplicate
- Determination of the effects of parameters on aroma production

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} X_i X_j$$

Simple effect
Quadratic effect
Interactions



Fermentation set-up

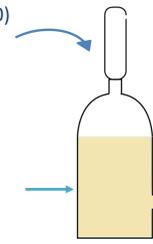
Yeast strains : *Metschnikowia pulcherrima* Flavia® (MP) et *Saccharomyces cerevisiae* Lalvin QA23® (SC)

250 mL of synthetic must (Bely et al. 1990) + thiols precursors

Thiol precursors' concentrations :
 Cys-3MH : 100 µg/L
 G-3MH : 500 µg/L
 Cys-4MMP : 10 µg/L
 G-4MMP : 10 µg/L

Inoculation :

- Sequential fermentation : MP at 1.10⁷ cells/mL + SC at 5.10⁶ cells/mL after 48 hours of fermentation
- SC (5.10⁶ cells/mL) in pure culture as a control.



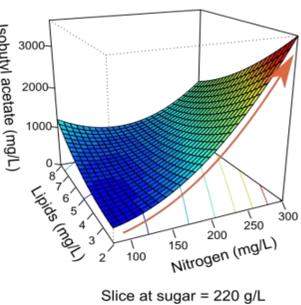
Samples at 90% of fermentation progress
 ➤ Aroma analysis by GC-MS

Impact on aroma production

Response surfaces :

- one parameter is fixed, the other two are represented on x and y axis
- the studied aroma is represented on the z axis

Acetate esters :

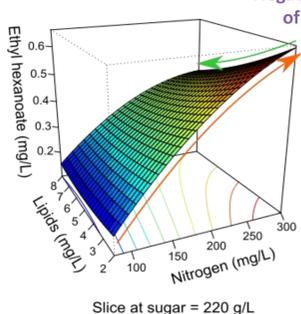


Positive effect of nitrogen

- Positive effect of nitrogen
- Interaction between lipids and nitrogen :
 - positive effect of lipids with low nitrogen concentrations
 - negative effect of lipids with high nitrogen concentrations

Regulation of ATF1 by both nitrogen and lipids

Ethyl esters :



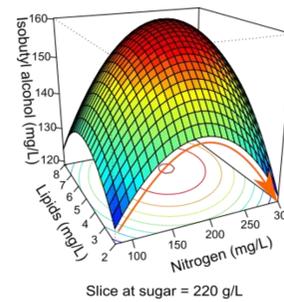
Negative effect of lipids

Positive effect of nitrogen

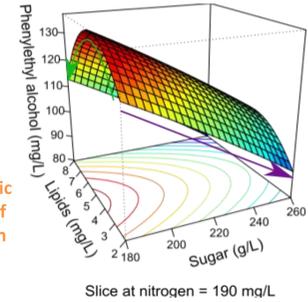
- Positive effect of nitrogen : already observed with *S. cerevisiae* (Torrea et al. 2011, Garde-Cerdan and Ancin-Azpilicueta, 2008)
- Negative effect of lipids

Repression of lipids biosynthesis and thus ethyl esters production by exogenous lipids (Chirala et al. 1992) ?

Higher alcohols :



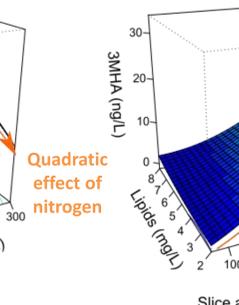
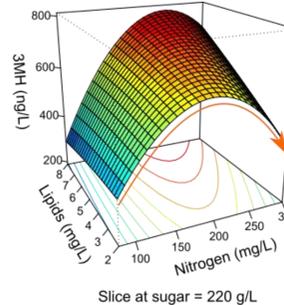
Quadratic effect of lipids



Negative effect of sugar

- Quadratic effect of nitrogen, as for *S. cerevisiae* (Rollero et al. 2015, Mouret et al. 2014)
- Except for phenylethyl alcohol : no significant effect of nitrogen

Thiols :



Positive effect of nitrogen

- Quadratic effect of nitrogen on the release of 3MH and 4MMP
- Positive effect of nitrogen on the production of 3MHA

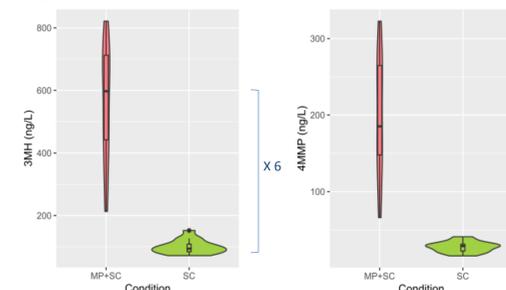
3MHA production using similar mechanisms than the other acetate esters

Comparison sequential inoculation vs pure culture

In sequential inoculation with *M. pulcherrima* Flavia® :

- Higher alcohols : ■ (except isobutyl alcohol)
- Acetate esters : + when **high** nitrogen concentration, ■ when **medium or low** concentrations
- Ethyl esters : ■
- Acids : ■

Thiols :



High production of thiols with MP
 ➤ *B*-lyase activity of MP ?

Condition
■ MP+SC seq. inoculation
■ SC pure culture

The comparison between sequential inoculation and pure cultures revealed the benefit of using *M. pulcherrima* in fermentation. Thanks to the Box-Behnken model, the impact and interaction of the parameters were assessed, showing that nitrogen was the most influencing nutrient on the sensory and organoleptic profile of wines.

These results are of great interest for the management of *M. pulcherrima* in sequential inoculation in alcoholic fermentation. They pave the way for further investigations on the metabolic and transcriptomic origins of the phenotypic specificities observed, and the interactions taking part between *M. pulcherrima* and *S. cerevisiae*.