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 esters and thiols) during *M. pulcherrima* / *S. cerevisiae* sequential fermentation.

### Materials and Methods

#### Fermentation set-up

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

**Inoculation** - Sequential fermentation : MP at 1.10^6 cells/mL + SC at 5.10^6 cells/mL after 48 hours of fermentation

**5.10^6 cells/mL** in pure culture as a control.

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

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

**250 mL of must**

**Samples at 90% of fermentation progress**

Aroma analysis by GC-MS

#### Experimental design

**Box-Behnken design**:
- 15 fermentations with central point in triplicate
- Determination of the effects of parameters on aroma production.

**Regression of ATRF by both nitrogen and lipids**

**Acetate esters**:
- 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

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

**Regulation of ATRF by both nitrogen and lipids**

**Thiols**:
- 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**

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*.

**Impact on aroma production**

**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

**Comparison sequential inoculation vs pure culture**

**Higher alcohols**: 
- Positive effect of nitrogen with low lipids
- Negative effect of lipids when high nitrogen concentration

**Acetate esters**:
- Positive effect of nitrogen when high nitrogen concentration
- Negative effect of lipids when medium or low concentrations

**Ethyl esters**:
- Positive effect of nitrogen

**Acids**:
- Positivity of the effect of nitrogen and lipids

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