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Yeasts potential interactions influencing the formation of fine aromas during cocoa fermentation process

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Context

Aromas of fine chocolate, **fruity, and/or floral notes**, might be partly linked to the surrounding environment of cocoa beans, (i.e. pulp) during fermentation (Chetschik *et al.,* 2017, Eskes *et al.,* 2012). Several works attempted to use selected yeast strains, known to produce higher alcohols and esters, to enhance the aromatic quality of chocolate. However, the **interactions between the different species** during fermentation are poorly documented, although they could provide relevant explanations for the success or failure of such procedure.

In this work, we use a **wine minimal medium (SM)**, modified to be closer to the cocoa pulp environment. This allowed us to focus exclusively on the formation of aroma compounds **from carbon and nitrogen sources by indigenous yeasts during fermentation**. Our findings show the significance of yeast interactions and provide clues on how yeast ecology can affect the formation of fine aromas originating from fermentation during the processing of raw cocoa beans. This represents a first step in designing rational strategies to **select yeast starters able to enhance and harmonize the production of fine chocolate**.

Objective

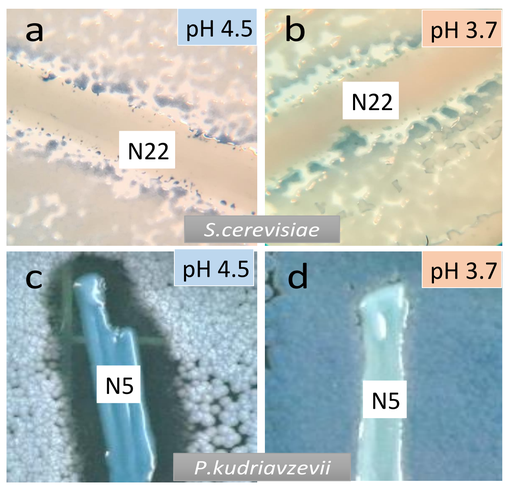
To study the interactions between main indigenous yeasts of cocoa fermentation and to understand how these interactions can influence the aromatic quality chocolate.

Materials and methods

- **Yeast strains :** 56 strains, isolated from 5 different batches (standard, high quality and premium) of fermented cocoa beans in Ivory coast : 19 *Saccharomyces cerevisiae* (S), 19 *Pichia kudriavzevii* (P) strains, and 18 other genera non-*Saccharomyces* (N) such as *Candida*, *Pichia*, *Hanseniaspora*, *Kluyveromyces,* or *Torulaspora*.
- **Interaction between yeasts strains : Killer assay**, YPD medium acidified with citrate phosphate buffer at pH 3.7 and pH 4.5 with methylene blue
- **Aroma compounds :** identification &quantification by DHS-SPME/GC-MS
- **Minimal pulp simulation medium** based on Belly *et al.,* 1990 (wine-derived medium : SM wine) 200 mg/L total nitrogen and N source provided equally by all amino acids, with some modifications (glucose 60 g/L, fructose 60 g/L, citric acid 8 g/L, pH 3,75). Five days fermentation with an initial inoculum of 10^6 cells/mL

Results and discussion

1 Interaction between yeasts strains : Killer assay



- No killer interaction or inhibition between *S. cerevisiae* and *P. kudriavzevii* strains.
- Weak negative interactions reflecting killer potential for ***Kluyveromyces marxianus* N22** against S strains at pH 3.7 and 4.5 and only at pH 4.5 for ***Wickerhamomyces pijperi* N48**.
- A very strong inhibitory effect at pH 4.5 of ***Torulaspora pretoriensis* N5** against *P. kudriavzevii*.

Figure 1 illustrates the interactions observed during killer assay between *S. cerevisiae* S56 and *K. marxianus* N22 (a,b), and *P. kudriavzevii* P66 and *T. pretoriensis* N5 (c,d). YPD medium acidified with citrate phosphate buffer at pH 3.7 (b,d) and pH 4.5 (a,c) with methylene blue.

2 Typical fermentation profile of *S. cerevisiae* and *P. kudriavzevii* in minimal pulp medium : Effect of initial oxygen concentration

The effect of initial oxygen content & availability was modulated by filling 10 ml container with 1 ml (high S/V, high oxygen) or 4 ml (low S/V, low oxygen) of medium.

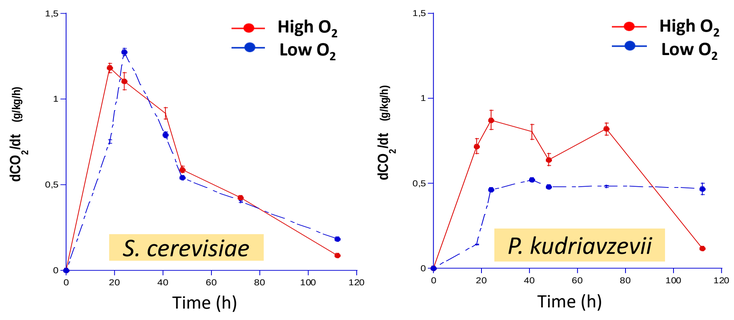
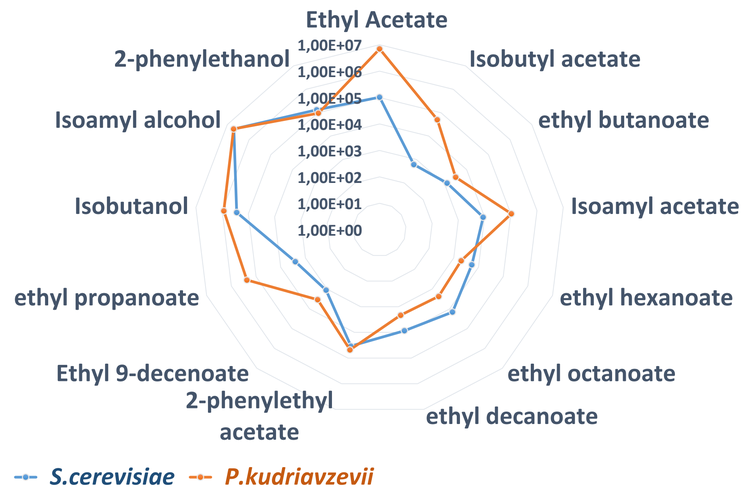


Figure 2 depicts the typical shape of the rate of CO_2 release [V_{CO2} (g/kg/h)] during 120 h of fermentation for *S. cerevisiae* and *P. kudriavzevii*

- *S. cerevisiae* had a typical behavior regarding V_{CO2} release with a maximum at the beginning of fermentation followed by a rapid decrease, whereas *P. kudriavzevii* showed a more singular behavior with a rather linear V_{CO2}.
- *S. cerevisiae* was a more efficient fermentative strains ($V_{CO2max} \approx 1.2$ g CO_2/kg/h) than *P. kudriavzevii* ($V_{CO2max} \approx 0.8$ g CO_2/kg/h).
- *S. cerevisiae* was only slightly influenced by O_2 content & availability, while this parameter had a strong effect on *P. kudriavzevii* fermentation kinetics, with a much slower V_{CO2}.

3 Fermentation on minimal cocoa pulp simulation medium : *S. cerevisiae* and *P. kudriavzevii* aroma production profile



- *S. cerevisiae* strains were characterized by the production of medium chain ethyl esters (ethyl hexanoate, octanoate, decanoate).
- *P. kudriavzevii* was highly represented in the production of acetate esters (ethyl, isobutyl, isoamyl acetate) and short chain ethyl esters (ethyl propionate).

Figure 3 : Aroma compounds production of all *S. cerevisiae* and *P. kudriavzevii* strains reflecting the typical profile of each species

4 Fermentation on cocoa pulp simulation medium : Intraspecific variability and interaction in coculture

S. cerevisiae (S) and *P. kudriavzevii* (P) species were well separated in 2 distinct groups with respect to their aroma production profile on cocoa model media (Figure 4).

With regard to *S. cerevisiae* (S) :

- S35, S38, S56, S77 & S60, isolated from premium & high quality batches, had close aroma profiles. S35 produced the highest concentration of 2-phenylethanol (floral and sweet note).
- *W. pijperi* (N48) had a very poor aroma profile production in comparison with all other strains, which can be explain by its low fermentation capacity (data not shown). N48 in coculture had almost no influence on the aroma profile of *S. cerevisiae* (S23; S68)
- *K. marxianus* N22 was a high producer of 2-phenylethyl acetate (fresh, fruity, pear note). N22 leads to an increase in the production of some esters when cocultured with S77, while the aroma profile was still close to S77 alone.

With regard to *P. kudriavzevii* (P) :

- Monocultures of *P* produced the highest amount of isobutyl acetate, ethyl acetate, ethyl proprionoate and isoamyl acetate (fruity note), particularly P76.
- Cocultures of *P* with other strains having killer effect (N5, blue outline) or not (S4 and S36, green outline) had a great impact on the degradation of the overall aroma profile of PK strain.
- S35 was also negatively affected in coculture with strain P36 leading to a global negative effect on aroma profile of both strains

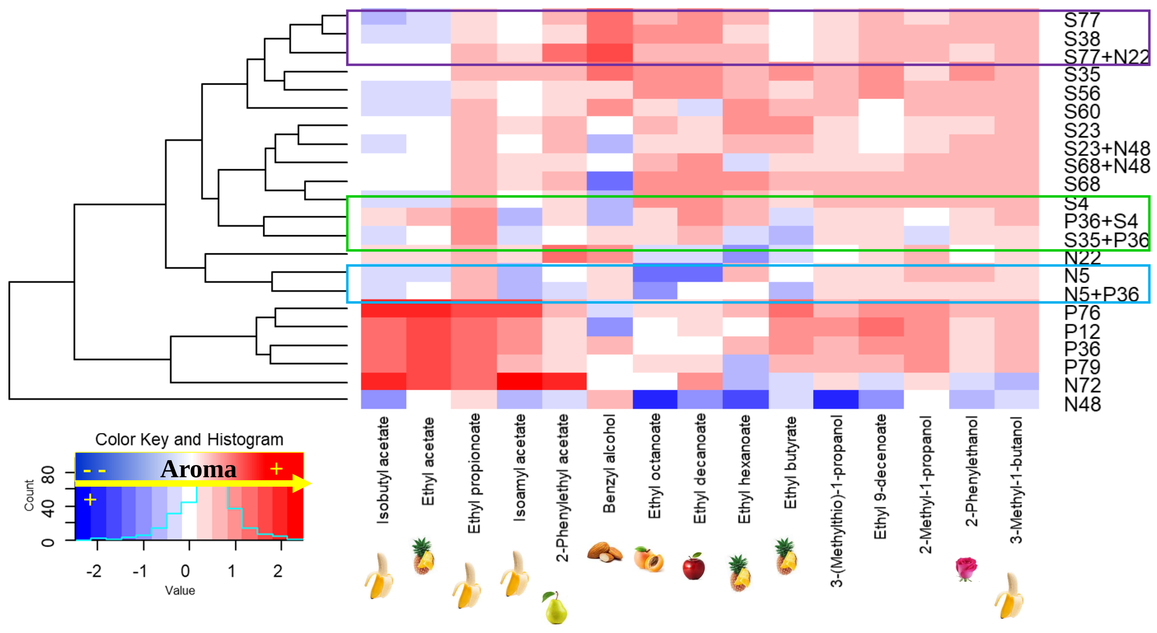


Figure 4. Heatmap summary of the aroma profile of cocoa pulp simulation media fermented by monoculture and coculture of *Saccharomyces cerevisiae* (**S**), *Pichia kudriavzevii* (**P**), and non-*Saccharomyces* (**N**) strains. **N22**: *Kluyveromyces marxianus*; **N48**: *Wickerhamomyces pijperi*; **N5**: *Torulaspora franciscae*; **N72 :***Pichia kluyveri*

Conclusion and perspectives

- Aeration of cocoa beans during fermentation in boxes might be a key parameter to favor the activity of *P. kudriavzevii* strains.
- *S. cerevisiae* is little affected in coculture but can benefit from the ability of *K. marxianus* to produce high level of esters.
- The aroma profile of *P. kudriavzevii* is strongly affected by the interaction with negative strain (N5) but also with *S. cerevisiae* strains.
- Two rich aroma producers, such as P36 and S35, cocultured together can lead to an impoverishment of the global aroma profile.

To come…

- ✓ Design a medium with cocoa-like amino acids profile and compare with real cocoa pulp matrix
- ✓ Amino acids preferences of indigenous yeasts
- ✓ Origin of the coculture effect: competition for aroma precursor / killer or inhibition effect/ esterase activities?

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