Code of good vitivinicultural practices in order to avoid or limit contamination by *Brettanomyces*

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PREAMBLE

Among processes that deteriorate wine quality, the production of volatile phenols by *Brettanomyce*s species is widespread and increasingly problematic. These compounds are characterised in particular by aromas of ink or glue, and horse sweat, leather or stable taints.

Volatile phenols, mainly 4-ethylphenol and 4-ethylguaiacol, are produced from p-coumaric acid and ferulic acid respectively after enzymatic decarboxylation (cinnamate decarboxylase, PAD) and reduction (vinylphenol reductase, VPR). These precursors are naturally present in grape musts. The decarboxylation step caused by cinnamate decarboxylase activity was described in many bacteria, yeast and fungi species, while the reduction step caused by vinylphenol reductase activity, or VPR, is more specific to the *Brettanomyces/Dekkera* species.

As *Brettanomyces* are present on grapes and on winemaking equipment, grape musts can be contaminated at a very early stage. However, these yeasts generally proliferate after alcoholic and/or malolactic fermentations, during wine maturing or after packaging.

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1. INTERVENTIONS IN THE VINEYARD

Not applicable (to our knowledge, no study available). However, *Brettanomyces* yeasts were detected on grape skins from the first stages of berry development. The microbial ecology of grape surfaces has shown great diversity, with a small population for each species (Renouf et al., 2007).

A first preventive approach, consisting of a rigoruous selection of healthy grapes, can play a role in reducing the risk of *Brettanomyces*, which is generally greater on rotten grapes.

2. INTERVENTIONS DURING THE GRAPE HARVEST

Grape management:

Brettanomyces is present on grapes but is not the major species of yeast (small population). However, the removal of rotten grapes could limit *Brettanomyces* spoilage.

The harvest of overripe berries is more and more common, and in this case particular precautions should be considered. The organoleptic impacts are interesting, but it increases the risk of volatile phenol production because overripe grapes contain more volatile-phenol precursors. Working in these conditions does not necessarily increase the presence of *Brettanomyces*, but increases the risk of activity (a lower total acidity and higher pH directly impacts on molecular SO_2 levels and consequently on the growth of *Brettanomyces*).

3. INTERVENTIONS IN THE CELLAR

Due to multiple factors, including the increase in alcohol content, a reduction in microbial diversity is observed during alcoholic fermentation. However, as *Brettanomyces* has good resistance to ethanol, its presence does not decrease, therefore perfect hygiene is essential during winemaking (sound grapes, winemaking and storage equipment, etc.).

3.1. Pre-fermentative operations and treatments

- Ensuring that suitable hygiene practices applied in the cellar is recommended.

- The most important factors are sulphiting and temperature:
  - sulphiting is the most effective preventative action at the pre-fermentative stage for limiting the development of *Brettanomyces* populations, however it is recommended that excessive sulphiting (>8 g/hL), which could delay malolactic fermentation, is avoided,
  - high-temperature, pre-fermentative maceration (above 65°C) results in the inactivation of *Brettanomyces*, but also of other microorganisms in winemaking. A cold maceration at a temperature lower than or around 10°C prevents their proliferation, but does not kill them.

- In all circumstances, subsequent contamination is possible.

3.2. Fermentation operations

Alcoholic fermentation (AF):

- During AF, microbial diversity decreases and *Saccharomyces cerevisiae* becomes the main species. However, due to its ethanol resistance and lower nutrient demand, *Brettanomyces* can grow as AF slows down or stops. The oenological practices commonly recommended for management of alcoholic fermentation must be implemented.
- Inoculation of musts with selected yeasts helps to achieve a more reliable AF.
- The environment becomes more favourable to the multiplication of *Brettanomyces* if alcoholic fermentation slows down or stops. In the case of the latter, using a process to restart alcoholic fermentation as soon as possible is recommended.
- Residual sugars (mainly glucose and fructose) are substrates for *Brettanomyces* growth. Wines are generally considered as dry when the sugar level is below 4 g/L. A concentration of 0.3 g/L of residual sugars is sufficient for the development of *Brettanomyces* biomass capable of producing over 1000 µg/L of volatile phenols.
- Yeast nutrients (that may also benefit *Brettanomyces*) should be added only if really necessary to avoid stuck fermentations.

Lag period before malolactic fermentation (MLF):

- Once AF is completed, conditions favour not only lactic bacteria but also *Brettanomyces*, although its proliferation remains slow.
- It is important to monitor the *Brettanomyces* population since the environment is relatively low in microorganisms.
- Factors favourable to the growth of *Brettanomyces* during this phase are: final high-temperature macerations (40-45°C), micro-oxygenation and the release of sugars in the case of uncrushed harvests.

– Co-inoculation of selected yeasts and selected lactic bacteria may help to reduce the lag phase between alcoholic fermentation and malolactic fermentation, and consequently the development of *Brettanomyces*.

Malolactic fermentation (MLF):

– Physicochemical parameters (pH, temperature, total SO_2) affect the progress of MLF. If MLF is delayed, the risk of production of volatile phenols increases because *Brettanomyces* can take benefit of this time to multiply.
– The use of malolactic starters is a good way to limit *Brettanomyces* development. Some studies have shown that co-inoculation or early sequential inoculation prevented *Brettanomyces* contamination by reducing the lag phase in between AF and MLF.
– After malolactic fermentation, it is recommended to eliminate all microorganisms particularly by adding SO_2 on its own or in combination with DMDC to obtain a synergistic effect (Renouf et al., 2008). These quantities must be adjusted according to the pH of the wine.

3.3. Maturation and clarification operations

The first necessary precaution is to carry out a complete microbiological analysis, which includes specific counting of *Brettanomyces*. This analysis must be repeated throughout the maturation period.

– SO_2 management is crucial to limit the development of *Brettanomyces*. The recommended concentration is about 0.5 to 0.8 mg/L of molecular SO_2[1].
– Ageing on lees is an additional risk factor because *Brettanomyces* are quite able to survive and to proliferate in lees (which release nutrients in wine).

[1] The final product should comply with the regulations in force regarding total SO_2 limits.

- Clarification by racking, fining and filtration are essential to reduce the viable and viable but non-culturable *Brettanomyces* populations which can multiply by metabolising residual sugars.
- Some fining agents are more efficient than others. Treatments with fining proteins can reduce populations by a factor of 40-2000. Fining using casein or potassium caseinate may reduce the ethylphenol levels if these are not too high.
- The addition of chitosan is one alternative for controlling the growth of undesirable microorganisms, particularly *Brettanomyces*.
- Some winemaking operations (racking, topping up, filtration, bottling, etc.) may result in oxygen dissolution in wine which favours the multiplication of *Brettanomyces*.
- If micro-oxygenation is practised, the absence of *Brettanomyces* should be checked using appropriate analyses.

NB:

1- Upon addition of SO_2, the *Brettanomyces* population can switch (fully or partially) from a viable state to a viable but non-culturable (VBNC) state. These changes lead to a reduction in the size of the yeasts, so it is necessary to adapt filtration.

2- It is also important to note here that enumeration of VBNC cannot be performed by routine analysis, for example enumeration on a Petri dish, but rather by qPCR or flow cytometry with in situ hybridization which enumerates VBNC and viable forms of *Brettanomyces* indifferently.

3.4. Barrel maturation

Barrel maturation is considered to be the period most sensitive to spoilage by Brettanomyces. During sampling, cross contaminations have to be avoided.

As for any microbial spoilage, the wine used for topping up must not be contaminated. Wood is favourable to growth of Brettanomyces, which is capable of using cellobiose as a carbon source. Barrels are difficult to clean and disinfect.

Old, poorly-cleaned barrels are known sources of contamination by *Brettanomyces.* However, new barrels also favour yeast multiplication and the production of volatile phenols, since they release more nutrients. Moreover, new barrels are more permeable to O2, which favours a relatively high redox potential and decreases the (active or molecular) SO2 concentration, two parameters favourable to Brettanomyces growth.

Different approaches have been investigated for the sanitization of barrels, but none of them have enabled the complete removal of Brettanomyces on the internal stave surface or bunghole. Indeed, the natural microporosity of the wood makes its complete disinfection difficult because microorganisms stayed alive in the cavities of the deep layers of the wood. A deeply-acting treatment is essential for long-lasting efficiency and results over time.

Nevertheless, some techniques for the disinfection of barrels significantly reduce Brettanomyces populations and may be used where permitted by the regulations of the country in question, for example:

- steam treatment: deep disinfection requires a sufficiently long treatment time (cold water rinse, hot water rinse at 70°C and low pressure steam for 10 min),
- ozone sanitization: either with gaseous ozone combined with a hot water treatment at 82°C for 20 min, or with ozonated water. By reacting with materials with a high organic load, the ozone does not deeply penetrate into the wood,
- SO_2 sanitization: a minimum of 5 g per barrel of gaseous SO_2 should be used to disinfect empty and dry barrels. SO2 is very efficient both on the surface and also in deeply penetrating the first millimetres of the wood,

- barrel shaving and re-firing: this treatment does not disinfect the wood but enables the most contaminated part to be removed. Shaving and re-firing enables an 80% reduction of volatile phenols compared to a non-treated barrel,

- ultrasound: this technique removes more than 90% of viable Brettanomyces (up to 2-4 mm below the internal stave surface).

3.5. Pre-packaging operations

The risk of volatile-phenol production must be evaluated before the packaging operations using analytical checks (both chemical and microbiological). When the risk has been evaluated, suitable operations must be designed in order to prevent post-packaging development of *Brettanomyces*:

- sterilisation by membrane filtration (0.45 to 1µm) or tangential filtration, for an efficient removal of *Brettanomyces* yeasts), followed by sterile packaging,
- use of DMDC for non-lasting protection,
- use of antimicrobials with lasting protection (sorbic acid, only if lactic bacteria have been removed completely, management of SO_2 taking the pH into account, etc.),
- heat treatment.

3.6. Storage conditions

In order to prevent Brettanomyces proliferation in bottles during storage (and the production of volatile phenols), keeping bottles below 12°C is recommended, especially for lightly-filtered wines or wines containing low levels of SO_2.

4. CONCLUSIONS

- Frequent analyses are highly recommended, in order to detect any contamination by Brettanomyces at an early stage. During sampling, particular attention must be paid to avoid cross-contaminations.
- The maintenance of the best possible hygiene conditions in the cellar is highly recommended.
- Sulphiting management.
- Temperature management.
- Preventive actions are preferable to curative processes.
- The present recommendations are based on current knowledge and are liable to be updated according to on-going research.