CHITOSAN AND BRETTANOMYCES: ORIGIN, IMPACT AND MODE OF ACTION

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Abstract

Certain forms of chitosan – a natural polymer of derived from chitin – present antimicrobial properties. Successfully extracting chitosan from a fungal source recently led to its utilization in oenology to fight against contamination with *Brettanomyces bruxellensis*. Different trials have shown that its utilization in wine at a rate of 4 g/hL was often sufficient to obtain the rapid and total mortality of contaminating cells. Other studies have revealed the existence of sublethal populations of *Brettanomyces* after treatment with chitosan, i.e., dying but not dead cells detected by certain analytical methods. These sublethal populations, incapable of redeveloping or of producing volatile phenols, are false positives that sometimes remain detectable in the wine for more than two months after treatment.

Research also supports hypotheses that chitosan's mode of action on *Brettanomyces* is both biological and physical. Electrostatic interactions between the *Brettanomyces* cell membrane and chitosan appear to be responsible for altering the integrity of the yeast membrane, leading to loss of energy and cell death, as well as for an adsorption phenomenon and the fining that accelerates the settling of the cells. These experiments show longer contact times can protect the wine during aging.

1. Introduction

Brettanomyces bruxellensis is a constant threat to the quality of wines. These spoilage yeasts are capable of developing in difficult environments, notably during the wine's aging phases.

Today, different means are used in the fight against *Brettanomyces*, some more successfully than others, but these means are not always sufficient. Accepted by the OIV (the International Organization of Vine and Wine) as an oenological practice since July 2009 and by the European Union since December 2010, fungal-source chitosan is an innovative tool in the fight against *Brettanomyces*. Several laboratory studies have shown the effectiveness of chitosan on *Brettanomyces*. Since 2008, winery trials confirm these results, and have led to determining the appropriate doses to use, while highlighting the existence of a "sublethal" state of *Brettanomyces* populations treated with chitosan. Knowing this has helped winemakers refine their methods for monitoring the effectiveness of the treatment, and has contributed to understanding chitosan's mode of action on *Brettanomyces* yeasts.

2. Chitosan

2.1 Origin of the chitosan utilized in oenology

Chitosan is a molecule derived from chitin. Chitin and chitosan are among the most widespread naturally occurring polymers on Earth, second only to cellulose. Chitin is found, notably, in the exoskeletons of crustaceans and insects, as well as in the cell walls of fungi.

A deacetylated derivative of chitin, chitosan is a β-1-4 linear copolymer of N-acetyl D-glucosamine and D-glucosamine (figure 1), obtained by subtracting acetyl groups (CH_3-CO). This operation frees the primary amino groups (R-NH_2) and confers a "cationic" nature on chitosan.

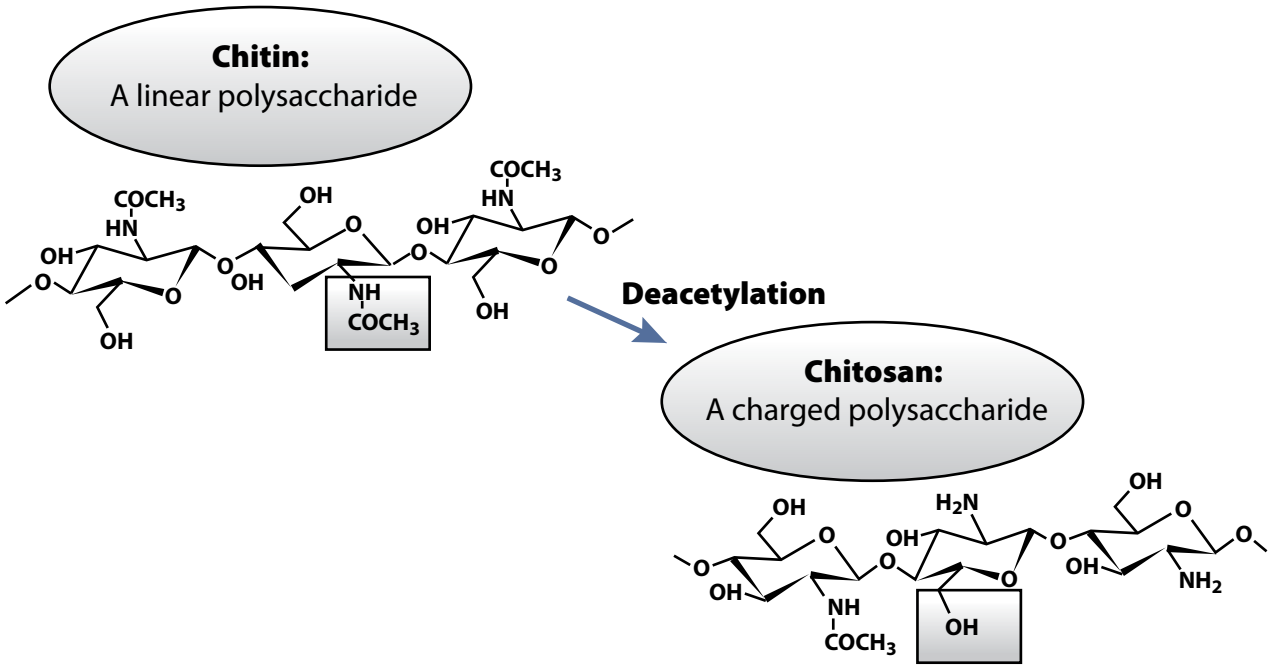

Figure 1. Chitosan, a polymer derived from chitin by deacetylation

In fact, there is not just one but several chitosans, as the variations in the degree of deacetylation, the molecular weight and the preparation of the formulations (granulometry, notably) result in molecules with varied properties and actions.

The innovation that led to receiving approval for utilizing chitosan in oenology is the process for obtaining chitin from a non-animal fungal source, *Aspergillus niger*. This process, patented by Kitozyme, provides natural-source chitosan that is biodegradable and non-allergenic.

Chitosan has been documented in several studies for its antimicrobial properties (Kumar 2000, Eaton et al. 2008, Kong et al. 2010, and Zakrzewska et al. 2005), which depend on, among other factors, its degree of deacetylation and its molecular weight (Zuehlke et al. 2013).

In a bioethanol co-fermentation with *Brettanomyces intermedius* / *Saccharomyces cerevisiae*, chitosan, at a dose of 2 g/L, was able to eradicate the *Brettanomyces* population without impacting the *S. cerevisiae*, thereby revealing some selectivity in the antimicrobial power of this polymer (Gómez-Rivas et al. 2004).

Bornet and Teisseidre (2008) then researched chitosan's oenological properties, notably vis-à-vis *Brettanomyces bruxellensis*, first in the laboratory then in vineyards of Languedoc-Roussillon (Blateyron-Pic et al. 2011). Ten days after chitosan treatment at a dose of 4 g/hL, the *Brettanomyces* populations in contaminated wines were generally no longer detected on selective growth gel media. At the same time, the same wines, untreated, often showed increased or maintained contaminating populations. Thus, the effectiveness of chitosan specifically in the fight against *Brettanomyces bruxellensis* in wine was clearly demonstrated.

2.2 Determining the dosage of chitosan

Wine designation	Brett Population at T=0	T=10 Control Population	T=10 Population No Brett Inside™ 2 g/hL	T=10 Population No Brett Inside™ 4 g/hL	T=10 Population No Brett Inside™ 8 g/hL
2010a Bourgogne Rouge	1.13×10^3	2.4×10^5	1×10^1	Absent	Absent
2010b Bourgogne Rouge	4.8×10^4	1.6×10^6	1×10^3	Absent	Absent
2010 AOC Village	1.5×10^4	2.5×10^5	Absent	Absent	Absent
2010 AOC Grand Cru	1.0×10^5	3.7×10^3	1.9×10^3	Absent	Absent

Figure 2. Trials on wines naturally contaminated with *Brettanomyces*

Population count on selective growth gel media of *Brettanomyces bruxellensis* at T=0 and T=10 days after treatment with No Brett Inside™ (pure chitosan) at different doses. Source: laboratory at IOC Nuits-Saint-Georges.

In laboratory experiments, Bornet and Teisseidre (2008) have shown that doses from 2 to 6 g/hL of chitosan were generally sufficient to completely destroy the *Brettanomyces* population, counted by quantitative polymerase chain reaction (qPCR) in a contaminated wine.

Laboratory experiments at IOC Nuits-Saint-Georges, in Burgundy, confirmed and refined this observation: in all cases, a 4 g/hL dose of No Brett Inside™ (a commercial product of pure, fungal-origin chitosan) was sufficient to totally decontaminate the wine of *Brettanomyces*, as counted on selective growth gel media (figure 2).

The experiments carried out by Jentzer in 2011 in the ENSIACET[1] chemical engineering laboratory furthered knowledge about determining the effective dose. In a synthetic model must contaminated with *Brettanomyces* at a concentration of 4×10^6 cells/mL, the effects of different doses (4, 10 and 40 g/hL) of chitosan were compared with an untreated control.

The counting method utilized was flow cytometry, which can count and differentiate live and dead *Brettanomyces* cells: the yeast cells stream by under the laser beam at great speed and are identified and characterized thanks to the utilization a dye. The cells marked red are alive and the green cells are dead. However, in the case of chitosan treatments, this technique showed the existence of cells coloured both green and red. Identified as *Brettanomyces* populations called "sublethal," the green and red cells correspond to dying cells whose membrane integrity has been altered.

By studying only the dead cell counts, this experiment showed that a maximum death rate of *Brettanomyces* is achieved in under five hours, but the death rate reaches only 60% (with a dose of 4 g/hL) to 80% (40 g/hL dose). Increasing the dose does not result in a quicker death. However, the total number of dead cells and sublethal cells reaches nearly 100% immediately after the chitosan is added, whatever the dose. In light of these results, it appears that the classic 4 g/hL dose is the one most often necessary and sufficient to reach maximum effectiveness within a few hours.

Next, we must ask whether the sublethal populations could make up a major portion of the yeast cells affected by the chitosan. Are these cells destined to die or are they capable of multiplying? Are they still capable of producing volatile phenols?

[1] École nationale supérieure des ingénieurs en arts chimiques et technologiques, part of the National Polytechnic Institute of Toulouse.

2.3 Sublethal populations and false positives

The sublethal populations of *Brettanomyces* already pose an analytical problem when qPCR controls are carried out. Although the flow cytometry technique allows us to visualize the ambivalent state of these cells, to date qPCR (a technique that amplifies DNA), has not allowed us to differentiate between living cells and sublethal cells. According to Chatonnet (2012), several days to several weeks can go by between the death of the cells and the loss of cell integrity, and the total disappearance of amplifiable DNA. As a result, in numerous cases the presence of false positive cells, i.e., cells detected as alive when they were in fact in the sublethal state, can be noted when a qPCR control is carried out after treatment with chitosan.

In the case of a control with selective growth gel media, it was often recommended to wait 10 days after racking the chitosan (which takes place 10 days after adding the chitosan product to the wine) before sampling and plating. When a control by qPCR was carried out, 10 days were generally shown to be far from sufficient.

Indeed, numerous experiments treating must with No Brett Inside™ followed by a qPCR control were carried out in Burgundy, Gironde and Spain (figure 3). We can readily see that certain populations detected as alive are detected as no longer alive only after 30 days or more.

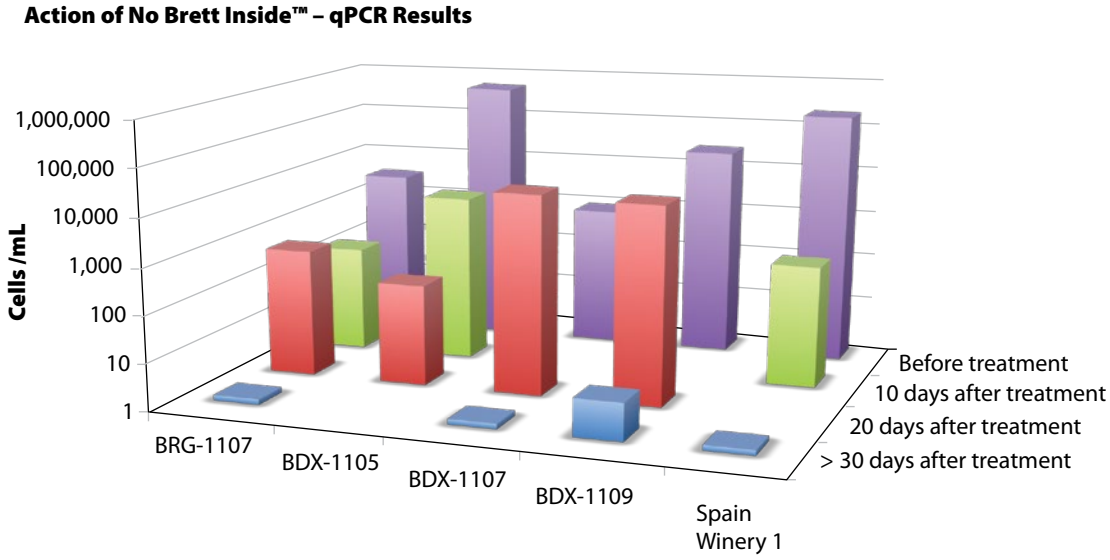

Figure 3. Evolution of the Brettanomyces population after treatment with No Brett Inside™ showing sublethal populations detected through quantitative polymerase chain reaction (qPCR)

The follow-up after treatment with chitosan compares counting results for different methods (figure 4). Although the *Brettanomyces* populations detected through qPCR remain very high after 40 and even 60 days, the counts on selective growth gel media show no *Brettanomyces* and the counts made by epifluorescence show that these high population levels correspond to dead cells.

		Cell count by quantitative PCR (UFC/mL)	Cell count by selective medium (UFC/mL)	Epifluorescence (UFC/mL)
Bordeaux Winery 1 (ref: BDX-1111)	Before treatment	>20,000		
	60 days after treatment	1.1 x 10^6	<10	1.1 x 10^6 dead cells
Bordeaux Winery 2 (ref: BDX-1108)	Before treatment	1.8 x 10^6		
	40 days after treatment	1.5 x 10^6	<10	5.7 x 10^5 dead cells

Figure 4. Comparison of the evolution of *Brettanomyces* populations after treatment with chitosan counted with quantitative polymerase chain reaction (qPCR), on selective growth gel medium or by epifluorescence by microscopy

We conducted further experiments to better analyze this bias inferred from the presence of cells in the sublethal state and the utilization of qPCR to monitor the effectiveness of the chitosan treatment.

In a vineyard in Gironde, we monitored two tanks of must contaminated by *Brettanomyces* and treated with chitosan at 4 g/hL for several months (figure 5). Once again, the results show very high *Brettanomyces* populations detected even two months after chitosan treatment. Indeed, the populations detected show major variations, like a sawtooth wave, that drew our attention. Thus, 45 days after treatment, the same wine samples were sent for qPCR counting in a second independent laboratory. The technique utilized by the second laboratory gave results that were much more regular and, moreover, detected much lower population levels of cells considered to be alive. Thus, the false positives detected appear to vary according to the qPCR technique utilized. In the end, these populations were no longer detectable.

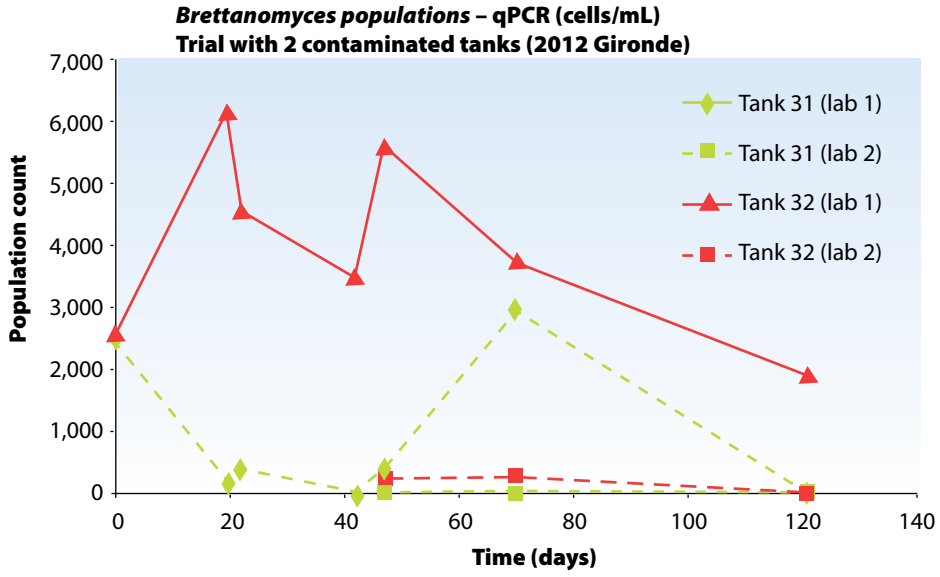

Figure 5. Long-term follow-up of *Brettanomyces* populations through quantitative polymerase chain reaction (qPCR) after treatment with chitosan, pointing out the presence of false positives

However, would these sublethal populations be capable of producing volatile phenols? To answer that question, we checked the concentrations of ethyl-4-phenol and ethyl-4-guaiacol in the must from both these tanks (figure 6). The levels measured at the end of the follow up were identical to the initial levels before treatment with chitosan. Therefore, these sublethal populations did not produce any volatile phenols. This result was confirmed by an experiment carried out in Spain (data not shown).

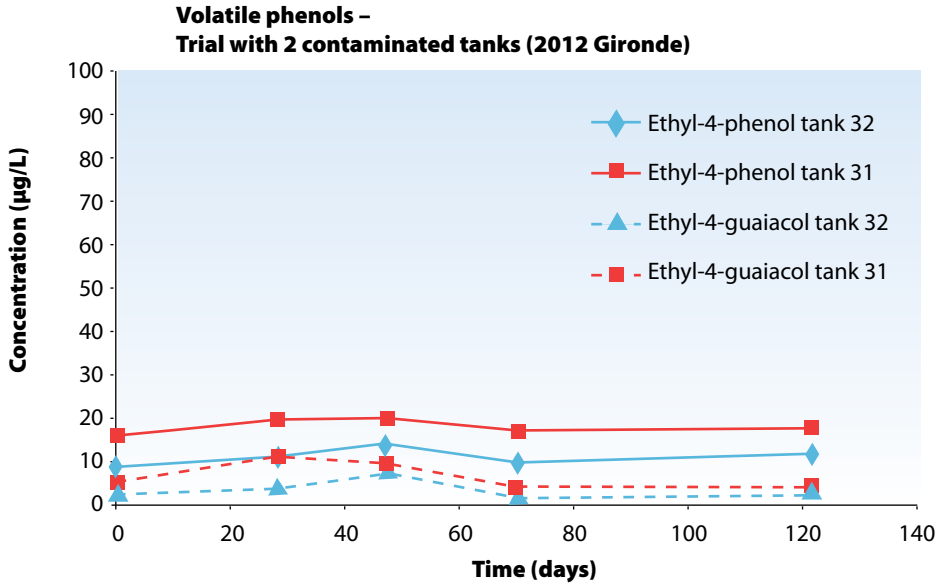

FIGURE 6. Long-term follow-up of volatile phenol production after treatment with chitosan, in the presence of false positives: pointing to the absence of volatile phenol production

From these experiments, three key points are important:

- The detection of sublethal populations strongly depends on the analysis method
- Sometimes, more time is required until these populations can no longer be detected; however, their existence is not necessarily systematic
- These populations no longer present any risk to the quality of the wine and they always end up dying.

2.4 How chitosan works

The scientific literature (Kong et al. 2010, Eaton et al. 2008, Zakrzewska et al. 2005, Sudarshan et al. 1992, Savard et al. 2002, Rabea et al. 2003, Zivanovic et al. 2004) abounds in hypotheses regarding the different interactions possible between *Brettanomyces* and chitosan, more generally on the antimicrobial role of this polymer:

- Given its cationic nature, chitosan could interact with the cell walls of microorganisms (electrostatic interactions)
- Chitosan could chelate the metallic ions necessary for microbial growth
- Chitosan could form a specific liaison with the microorganism's macromolecules (e.g., proteins, electrolytes, DNA, etc.).

This led Jentzer (2011) to formulate a hypothesis: electrostatic interactions could be formed between the chitosan and the cell walls of the microorganisms, as well as adsorption phenomena causing the death of the yeast cells.

Different observations support this hypothesis. Blateyron-Pic et al. (2012) have visualized through a classic microscope and an electron microscope that scans cells before and treatment with chitosan. These observations highlight the physical action of chitosan: the *Brettanomyces* cells are adsorbed and agglomerate around the chitosan particles and then flocculate. The particles can then be eliminated by racking.

However, these interactions appear to go further than the simple fining of *Brettanomyces* by chitosan. In fact, these authors followed up with adenosine triphosphate (ATP) testing of a synthetic must contaminated with *Brettanomyces* (at a level of 18 x10^6 cells/mL) then treated with chitosan (10 g/hL), comparing this must with the same contaminated but untreated must. This means of salting out intracellular ATP was carried out for two hours and showed that the presence of chitosan generated a release of the ATP in the medium. This phenomenon reveals a strong disturbance in the permeability of the *Brettanomyces* membrane that very probably correlates with the *Brettanomyces* mortality observed.

Which of the two effects of chitosan, biological or chemical, is the most important vis-à-vis the elimination of *Brettanomyces*? If fining were the main effect of chitosan, could other fining techniques be as effective at eliminating *Brettanomyces* populations?

To answer these questions, we carried out experiments in the laboratory, comparing a control wine naturally contaminated with *Brettanomyces* to the same wine treated with No Brett Inside™ at a rate of 4 g/hL and to the same wine treated only with two gelatines (A and B) recommended in the field in the fight against *Brettanomyces*, according to the prescribed dosage. Sixty-five days after treatment, qPCR testing was carried out and clearly showed the difference between a treatment with chitosan and a treatment with gelatine (figure 7).

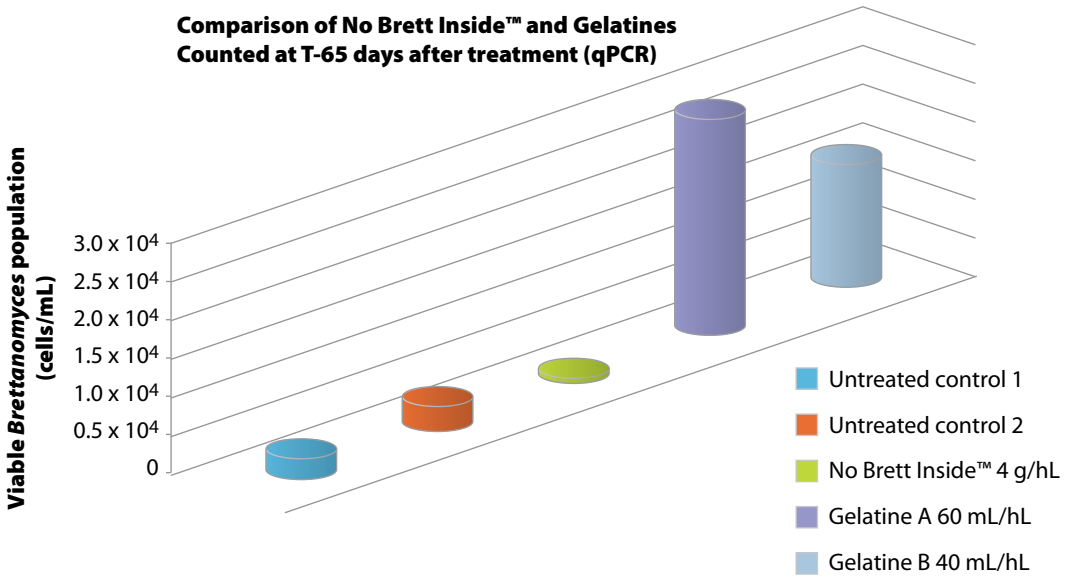

FIGURE 7. Highlighting the combined biological and physical effects of chitosan compared to the effect of fining, shown here by the utilization of gelatine

Alhough the *Brettanomyces* population dropped in the control wine, this decrease is much greater with the utilization of No Brett Inside™. In comparison, after a partial elimination phase of the contaminating population, the gelatine treatments caused a major regrowth of the contaminating population. This regrowth does not consist of false positives: the follow-up of the volatile phenol concentrations shows that the *Brettanomyces* populations indeed produced volatile phenols. Very little volatile phenol production was found in the control, and absolutely none was found in the wine treated with No Brett Inside™ (figure 8).

What these experiments clearly show is that chitosan's combined physical and biological effects on *Brettanomyces* ensure its effectiveness.

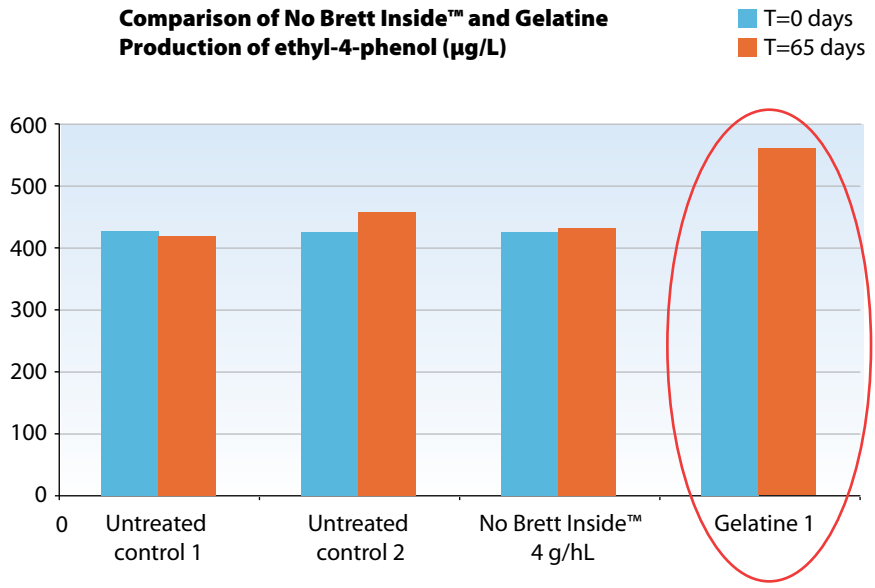

FIGURE 8. Highlighting the absence of volatile phenol production after treatment with chitosan and after fining with gelatine

These findings lead to the recommended protocol for utilizing chitosan. Related to the close contact between the cationic polymer and the *Brettanomyces* cells, chitosan's effectiveness depends on its homogenous incorporation into the wine, which begins by suspending the chitosan product in water or wine, then adding it to the tank to top off the total volume of the liquid to be treated. The use of fining couplings, which can impair the effectiveness of the treatment, should be avoided. In cases where the protocol is utilized on wine in barrels, the winemaker must be careful during *bâtonnage* to not incorporate too much oxygen. If a volume of wine must be removed from the container to allow a topping-off treatment without spill-over, this volume (which is still contaminated) must also be treated before it is returned to the tank. The winemaker must then wait at least 10 days before racking the wine to separate the chitosan and the *Brettanomyces* it has adsorbed.

3. Perspectives: Chitosan during wine development

These new findings on chitosan's mode of action open new horizons to explore. For example, when the wine develops over a long period, the winemaker may be interested in a longer contact time with the chitosan, and delay racking more than 10 days after incorporation. The advantages will double:

- Instead of carrying out an unnecessary racking, the winemaker can take advantage of classic racking to eliminate the product
- Above all, delaying racking could protect the wine from possible recontamination with *Brettanomyces*.

An experiment was carried out in Italy by Sieczkowski and Nardi (2012) to prove these hypotheses and optimize the utilization of chitosan. A Merlot wine and a Sangiovese wine were each inoculated with *Brettanomyces* (10^3 cells/mL) and each wine was separated into three aliquots:

- An untreated control, which underwent *bâtonnage* once per week
- Two aliquots per wine treated with No Brett Inside™ (4 g/hL), with no racking; one underwent *bâtonnage* once per week, the other did not undergo *bâtonnage*.

Each wine in the trial was monitored over six months. The SO_2 of all the wines was readjusted after 200 days.

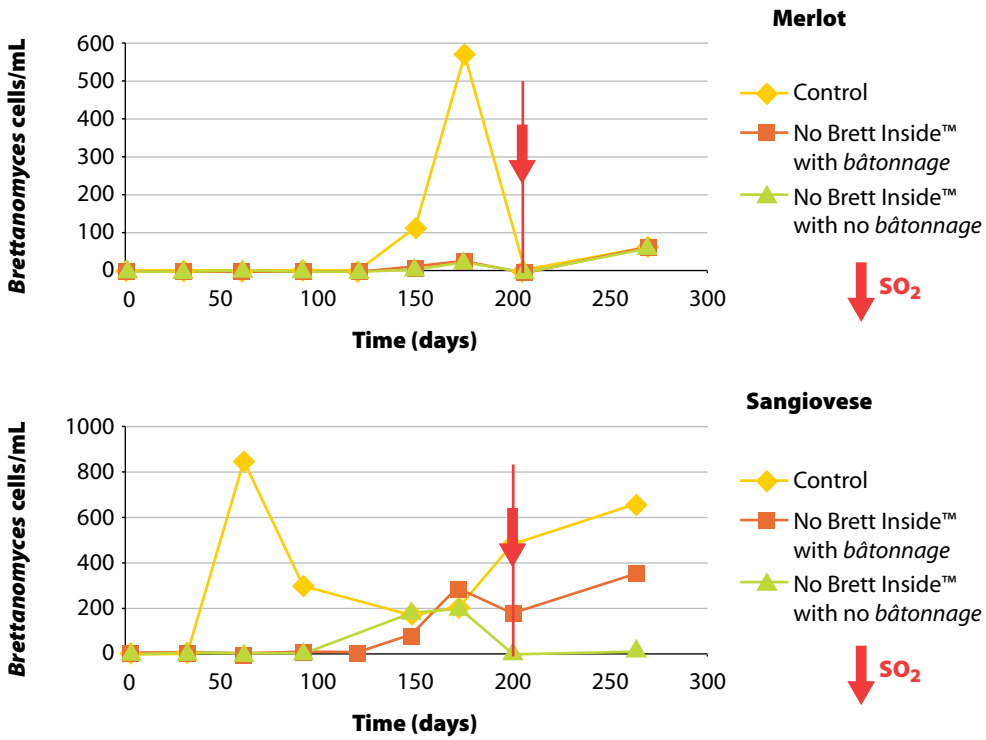

FIGURE 9. Prolonged contact between wines and chitosan, with or without *bâtonnage*, showing the impact of protecting the wine against *Brettanomyces*

The results (figure 9) seem to show the value of prolonged contact with chitosan while the wine is developing. However, the method with no *bâtonnage* appears to be most effective with the Sangiovese wine, although it does not encourage repeated contact between the product and the yeast cells. These trials do not allow us to come to a clear conclusion, but we can imagine two explanations:

- The *bâtonnage* encouraged the regrowth of *Brettanomyces* by putting the lees back in suspension, incorporating oxygen, etc.
- The *bâtonnage* resulted in a bias in the estimation of the *Brettanomyces* concentration in the wines, by re-suspending and homogenizing the cells.

The important role of SO_2 must also be noted, as it is a tool for control that remains necessary and acts in synergy with the action of the chitosan.

Additional research aimed at completing and refining these conclusions is underway.

3. Conclusions

In light of the experiments carried out by various research teams, as well as in our laboratories and in the field, we can confirm that pure, fungal-source chitosan is of great interest in the battle against *Brettanomyces* contamination.

The homogenous incorporation of chitosan into the wine, a guarantee of its effectiveness, results in the total destruction of the *Brettanomyces* populations, or, in certain cases, a significant reduction of the contaminating populations.

The winemaker must be careful to ensure a control protocol adapted to these treatments, considering the existence of sublethal populations and the detection of false positives by certain counting methods.

Much research has clarified chitosan's mode of action vis-à-vis *Brettanomyces*, while highlighting the speed of its action, both for reducing the population and preventing the appearance of volatile phenols. Thus, monitoring the ethyl phenol levels remains a complementary method to ensure and validate the implementation of the treatment.

Non-allergenic, naturally sourced and with no negative impact on the sensory quality of wine, fungal-sourced chitosan appears to be an innovative and unique tool to avoid the loss of quality generated by *Brettanomyces bruxellensis*.

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