Evaluation of plant phenolic extracts as an alternative to sulfur dioxide for the control of *Oenococcus oeni* and *Brettanomyces bruxellensis*

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Abstract. In the current work, the potential of plant phenolic extracts to replace the ability of SO_2 to inhibit spoilage microorganisms was evaluated. The study included the application of different doses of plant phenolic concentrates to test their antimicrobial activity against *O. oeni*, and *Brettanomyces* yeasts. For the evaluation of *O. oeni*, Chardonnay and Riesling wines were inoculated with the lactic acid bacteria, and antimicrobial agents were added: SO_2 or phenolic concentrates. Then within seven weeks and six months, the levels of malic and lactic acids in the control and treated samples were monitored. In the tests to verify the control of *Brettanomyces*, oak cubes were contaminated with the spoilage yeasts and later treated with water (control) or two aqueous sanitizing solutions (conventional one: water with citric acid and SO_2; experimental one: containing phenolic concentrates). Later, the corresponding solutions and water were analyzed for the presence of *Brettanomyces* yeasts by observation and plate numbering. Results confirm the inhibitory capacity of the phenolic concentrates tested regarding these two microorganisms, showing promising results.

1 Introduction

Sulfur dioxide (SO_2) has been used as a preservative and antimicrobial agent in winemaking for centuries, and has acquired relevant importance throughout all the winemaking processes thanks to its properties, such as antioxidant, antioxidasic, antiseptic, and disinfection of wine vessels [1]. SO_2 is an effective tool to maintain the quality and stability of wines by inhibiting the growth of undesirable microorganisms such as bacteria and yeasts. These microorganisms can significantly impact wine quality by causing spoilage and off-flavors [2]. The antimicrobial activity is caused by the destruction of disulfide bonds in regulatory proteins and enzymes, as well as reacting with nucleic acids and lipids, thus affecting the microorganism's membranes [3,4]. Despite its useful properties, it is considered a potentially toxic compound and may cause adverse reactions for wine consumers in quantities above 10 mg/L [5], and in some people, it can induce adverse clinical effects such as dermatitis, urticaria, flushing, hypotension, abdominal pain, diarrhea, and life-threatening anaphylactic and asthmatic reactions [6]. Consumers also commonly associate sulfites in wine with headaches, although the effect is stronger in subjects who are already sensitive to this problem [7]. In light of this, the World Health Organization (WHO) and the International Organization of Vine and Wine (OIV) recommend encouraging research on alternative methods of preservation aimed at reducing the use of SO_2, however, wines made with reduced SO_2 levels must not compromise the quality of the wine in terms of sensory characteristics and microbiological stability [5,8].

Malolactic fermentation (MLF) is a procedure that is recommended mainly for red wines, some white wines, base sparkling wines, and certain fruit wines [9]. MLF may not be advisable in all regions or styles of wines as the impact on the aroma can be positive or negative, depending on the nature and concentration of the aromatic compound. An example of a compound is diacetyl, which can contribute to the sensory profile of wine with a buttery aroma, which may be desired or unwanted depending on the style of wine. Other examples may be acetaldehyde or esters. For these reasons, another methodology must be considered to stabilize wines containing malic acid, which is a readily biodegradable molecule [1,10]. MLF in wines is often unpredictable and difficult to control or manipulate, which can compromise quality by the formation of off-flavor compounds (acetic acid, volatile phenols, and viscosity, among others), and can even produce compounds hazardous to human health (such as ethyl carbamate and biogenic amines). For this

reason, it is essential to control this biochemical process during winemaking to ensure the final quality of the wines [9,11]. *O. oeni* is the predominant lactic acid bacteria (LAB) species at the end of alcoholic fermentation. This species is the best adapted to grow in difficult conditions imposed by the medium and it is, therefore, the main species responsible for MLF in most wines [11,12]. In spontaneous fermentations, at the beginning of the alcoholic fermentation, the concentration of LAB is around 10^3 CFU/mL. Once the first fermentation is over, bacteria develop a lag phase, and once the growth starts, their concentration rises at 10^6 to 10^8 CFU/mL, at this concentration, they can start the MLF [13]. Among the parameters that affect the development of *O. oeni* are pH, SO_2, temperature, content of oxygen, organic acids, nutrients, lysozyme or chitosan residues, oenological and cellar practices and, finally, phenolic compounds. In oenology, studies have been carried out with different plant extracts, and with several types of phenolic compounds, by different authors seeking to replace or at least reduce the doses of SO_2 used for control of lactic acid bacteria [14-18] and of spoilage yeasts [23,24]. The inhibition mechanism involves phenolic compounds reacting with bacterial cell membrane proteins by increasing permeability and causing a loss of important components such as potassium, glutamic acid and intracellular RNA, as well as altering the fatty acid composition of the cell membrane or the inhibition of extracellular microbial enzymes [19-22]. The mechanisms of action against yeasts involve different effects such as disruption of the plasma membrane, inhibition of cell wall formation, dysfunction of mitochondria, inhibition of cell division, efflux pumps and the synthesis of RNA and DNA [25].

Brettanomyces spp. is a yeast species found in many wine-producing countries [26], and is often considered as a spoilage microorganism. It can produce volatile phenols with unpleasant aromatic notes and in the presence of free oxygen, produces high amounts of acetic acid (from 1,4 to 8,4 g/l) [27]. This yeast is also associated with the production of toxic biogenic amines, the hydrolysis of anthocyanins followed by the loss of wine color, and the production of tetrahydropyridines from lysine, acetic acid, guaiacol and various ethyl esters from short-chain fatty acids [28] which is considered as one of the main defects of red wines and is associated with important economic losses [29,30]. The high resistance and adaptation to stress conditions such as high ethanol concentrations (up to 15%), low pH (3), low sugar content (less than 300 mg/L), low oxygen transfer rates (less than 24 mg O_2 L^{-1} h^{-1}), low nitrogen levels (below 6 mg/L), and its tolerance to commonly used doses of SO_2, make this yeast one of the major microbiological problems to control in wineries [26,30,31]. The ability of *Brettanomyces spp*. to form a biofilm favors the adherence of cells to various types of surfaces and creates a protective layer, explaining its frequent presence in oak barrels. In oak vessels it has been found 8 mm deep in the wood staves, surviving around of the bung holes, in the structure of the oak and in the yeast lees [32]. The usual practice of conserving barrels and disused wooden vessels, which consists of filling these containers with a solution of acidified water with SO_2 (chlorine-free water, citric acid or tartaric to ensure pH <3.0, and an SO_2 addition of 250 mg/L) [33,34], creates an additional environmental problem.

Searching for natural alternatives in the substitution or reduction of SO_2 in winemaking, this research work evaluated the use of plant phenolic extracts with the specific objective of having an alternative tool for the control of malolactic fermentation and to control the development of *Brettanomyces,* including sanitizing oak surface, which are aimed to be in contact with wine (barrels, staves, etc.).

2 Materials and methods

2.1 Product of phenolic concentrates

The phenolic substances used are a mixture of natural tannins extracted from multiple plant materials. Its production is carried out under the trade name “Estaan”, which is in the process of being approved (Biolethics Europe, Netherlands). The botanical formulation originates from a sequential multi-stage separation technique, followed by several purification steps, and stabilized in a pH-buffered water/ethanol solution. The concentrate originates from ten different plants (*Rosmarinus officinalis, Vaccinium myrtillus, Morus alba, Melissa officinalis, Ananas comosus, Mangifera indica, Punica granatum, Prunus domestica, Lycium chinense fructus and Ruta graveolens)*. The following lot numbers of Estaan product were used: 01J2103 for the experiments with *O. oeni;* 20J2201 for the experiments with *Brettanomyces.*

2.2 Control of *Oenococcus oeni*

Two modalities were developed in the tests to investigate the anti-microbial properties of phenolic plant extracts against *O. oeni,* differing on the essay scale. The levels of malic and lactic acid were monitored after the controlled inoculations of commercial bacteria. The commercial product Bi-Start Vitale SK11 was used as a malolactic starter (Erbslöh, Germany). This is a freeze- dried *O. oeni*, with a number of vital cells $>10^{11}$ CFU/g according to the information of the producer. The inoculated dose (1g/hL) was the one recommended by the manufacturer, resulting in 10^6 CFU/mL. The selection of this bacterial strain was based on its ability to survive at the following wine conditions (pH > 3.0; free SO_2 < 15 mg/L; total SO_2 tolerance of 50- 60 mg/L; temperature > 16°C; Alcohol < 15.5% alcohol by volume) [35].

First, laboratory scale tests were carried out with different formulas of phenolic compounds and at different doses. A Chardonnay wine (2019 harvest) produced in the experimental winery of the Hochschule Geisenheim (Germany) was used for these tests. The alcoholic fermentation was carried out in stainless steel tanks using the commercial yeasts Oenoferm Freddo (Erbslöh,

Germany) at a dose of 30 g/hL, without the addition of SO_2 during vinification. Once alcoholic fermentation was finished, the wine was filtered by depth filtration with K100 and EK filter sheets (Pall, Bad Kreuznach, Germany). The basic parameters of the of the initial wine were recorded by Fourier transform infrared spectroscopy (FTIR), and are presented in Table 1. The values of malic and lactic acid, 4,5 g/L and 0,3 g/L, respectively, which indicated that no malolactic fermentation took place during one year of wine storage.

Table 1. General Chardonnay wine analysis. All the values are means ± standard deviation of three replicates.

pH	Total acid. g/L	Malic acid g/L	Lactic acid g/L	Ethanol g/L	Total SO_2 g/L	Free SO_2 g/L	Total phenols mg/L
3,2 ± 0,0	9,2 ± 0,0	4,7 ± 0,0	0,3 ± 0,0	97,7 ± 0,1	11,0 ± 0,1	2,0 ± 0,1	117,0 ± 0,0

Before inoculating with *O. oeni*, and to guarantee that the wine had the necessary nutrients for completing the malolactic fermentation, the product 'Bi-Start Nutri' (Erbslöh, Germany) was added at dosage of 20 g/hL. Subsequently, the wine was distributed in 300 mL graduated cylinders (previously sterilized), with 250 mL of wine each, and then placed in the Variomag Multipoint® magnetic stirrer. The phenolic concentrates were applied in three concentrations, one, two and three mL per 1 L of wine, named low phenols (LP), medium phenols (MP) and high phenols (HP), respectively. The phenolic levels of the treatments were corroborated by measuring the total phenols by the Folin-Ciocalteu, yielding values of 182 ± 1 mg/L for the LP treatment , 211 ± 0 mg/L, for the MP treatments, and 306 ± 1 mg/L for the HP treatments. Two control treatments were used, one without anti-microbial agents and another treatment with SO_2 molecular target level of 0,3 mg/L to have an inhibitory effect on bacterial growth [36], by adding a 5% SO_2 water solution. The malic acid concentrations were determined by enzymatic assay (Roche Boehringer Mannheim/R-Biopharm, Darmstadt, Germany) and all treatments were performed in three replicates.

Once the experiment on the laboratory scale was finished, the test was extended to larger volumes. Two trials were performed vinifying two Riesling musts, one from healthy grapes and the other from grapes affected between 50% and 70% incidence of *Botrytis* bunch rot. For each of the trials, three treatments of the must were carried out: a control without preservatives; a control with SO_2 (5 g/hL of SO_2); and addition of the phenolic concentrate (2 mL/L). After pressing, each of the two trials followed the following protocol: 600 liters of must were placed into a 1000 L container, homogenized, transferred into three 200 liters and the additives (SO_2 or phenolic concentrate) were added according to the treatment. Then the containers with must were placed in a cold room at 8°C for two days, for sedimentation. Once the clean must was obtained, it was transferred to 50 L containers, in three replicates per treatment, where they were inoculated using the commercial yeasts Oenoferm Freddo (Erbslöh, Germany) at a dosage of 30 g/hL. Once the alcoholic fermentation was finished, the free SO_2 levels were adjusted to 20 mg/L for the control treatments with SO_2, while a dose of 3 mL/L of phenolic concentrates was added to the phenolic treatments. All treatments subsequently received an inoculation of lactic acid bacteria.

Basic oenological parameters, and total phenols are presented in Table 2 after the additions. All the wines were kept for six months in stainless steel containers, then filtered by depth filtration with K100 and EK filter sheets (Pall, Bad Kreuznach, Germany), and bottled. At the time of bottling, all oenological parameters were controlled again, checking if no MLF took place after six months of tank storage.

Table 2. General wine analysis. All the values are means ± standard deviation of three replicates.

Riesling wines from botrytised grapes

Treatment	Control	SO_2	Phenols
Alcohol g/L	93,80 ± 0,10	93,70 ± 0,10	95,90 ± 0,00
Total acidity g/L	9,90 ± 0,00	10,30 ± 0,00	10,30 ± 0,00
pH	2,80 ± 0,00	2,80 ± 0,00	2,80 ± 0,01
Malic acid g/L	3,50 ± 0,00	3,87 ± 0,06	3,70 ± 0,00
Lactic acid g/L	0,30 ± 0,00	0,30 ± 0,00	0,20 ± 0,00
Free SO_2 mg/L	0,00 ± 0,00	20,00 ± 0,58	0,00 ± 0,00
Tot SO_2 mg/L	19,67 ± 0,58	102,00 ± 1,15	18,00 ± 0,00
Tot phenol mg/L	151 ± 0,00	233 ± 1,00	274 ± 0,00

Riesling wines healthy grapes

Treatment	Control	SO_2	Phenols
Alcohol g/L	89,10 ± 0,10	88,50 ± 0,10	90,87 ± 0,06
Total acidity g/L	10,23 ± 0,13	10,17 ± 0,06	9,70 ± 0,00
pH	2,80 ± 0,06	2,80 ± 0,00	2,80 ± 0,00
Malic acid g/L	3,32 ± 0,05	3,43 ± 0,06	3,33 ± 0,05
Lactic acid g/L	0,08 ± 0,10	0,40 ± 0,00	0,30 ± 0,00
Free SO_2 mg/L	2,75 ± 0,05	28,00 ± 0,00	2,67 ± 0,58
Tot SO_2 mg/L	8,25 ± 0,58	89,10 ± 1,15	12,00 ± 0,00
Tot phenol mg/L	117 ± 0,82	165 ± 0,00	284 ± 0,00

2.3 Control of *Brettanomyces sp.*

To evaluate the efficiency of the phenolic concentrates, reactivated cultures of *Brettanomyces* were inoculated to a Merlot wine to which oak cubes had been introduced. These cubes were then placed in antimicrobial solutions with a control treatment without microbial agents (only water), a control treatment with citric acid and SO_2, and a treatment with phenolic concentrates. Subsequently, the solutions exposed to the contaminated cubes were placed in Petri dishes for incubation, where the development of colonies that could survive the treatments was quantified. The additional step, which involved the use of cube-shaped oak, was aimed at evaluating whether microorganisms could be transferred from wood to culture media, and to evaluate the effectiveness of an aqueous solution with plant phenolic compounds as a sanitizer for oak barrels, staves, etc.

The test was carried out in the microbiology department of the Hochschule Geisenheim University. *Brettanomyces bruxellensis* yeast genus, 21B37 strain, extract from the yeast collection of the university, was used. The yeast culture stored frozen at -80 °C was reactivated and inoculated into YPD (yeast peptone dextrose) broth in a Petri dish for further growth. After

three days, one of the colonies was transferred into 100 mL of YPD liquid medium and placed in the Innova 4230 special shaking incubator at 25 °C and 130 rpm within three days. For yeast growth, the composition of YPD broth corresponds to glucose 20 g/L, peptone from Casein (by Carl Roth), yeast extract bacteriological (by VWR Life Science), and agar 20 g/L. Yeasts grown on the YPD broth were separated from the medium by centrifugation on the Eppendorf 5804R at 3.000 rpm and 22 °C for 5 min, then 10 mL of PBS (phosphate buffered saline) were added to carry out washing. Cell concentration in the suspension obtained from this process was counted with Goryaev-Thoma's method [37]. The *Brettanomyces* cell number was calculated by the following formula:

$$x = \frac{(N{:}4)\cdot 10^4}{0.04} = \frac{(491{:}4)\cdot 10^4}{0.04} = 3.069 \cdot 10^8 \text{ (cells per L)} \qquad (1)$$

The suspension obtained previously was mixed and seeded on the solid culture media in Petri dishes under aseptic conditions (in laminar flow boxes) using only sterile laboratory equipment and utensils, for colony multiplication.

Once the yeasts were reactivated, a sterilized base wine was used as initial culture medium. The wine was initially vinified and kept in a 25 L stainless steel tank, and then filtered by depth filtration with K100 and EK filter sheets (Pall, Bad Kreuznach, Germany), to later be bottled. The bottles underwent a pasteurization process, being submerged in a water bath at 65 °C for five minutes, with the aim of reducing microorganism populations. Conventional analyzes were performed including FTIR demonstrating the following general parameters: 13.2% of ethanol (v/v); 0.3 g/L of residual sugars; 6.5 g/L of total acidity; pH 3; 2 mg/L of free SO_2; 8 mg/L of total SO_2. The Merlot wine was not subjected to MLF.

In a 3 liters conical flask, 750 mL of the Merlot wine and 750 mL of a water solution with glucose and fructose were placed, in order to create a friendly media for *Brettanomyces* yeasts. The concentration of sugars in the wine solution was 10 g/L of glucose and 10 g/L of fructose. Then 10 ml of the sample *Brettanomyces* yeast culture was placed in the flask, well mixed and 100 µL of the suspension was immediately pipetted and inoculated into the Petri dishes at the following dilutions: 10^1, 10^2, 10^3, 10^4, to count the number of microbial contaminants. The yeast cells number after the inoculation were 53^5 CFU/mL of suspension, quantified with the Koch's method of plate counting [38]. Later 21 oak cubes (± 2 cm x 2 cm 1.5 cm, Tonelería Nacional, Chile) with a medium level toast, were introduced into the flask with the wine solution and kept at room temperature for 3 days.

To check and compare the antimicrobial power of the phenolic concentrates, six treatments were prepared: a control solution without additions of antimicrobial agents (only deionized water), a conventional sanitizing solution (deionized water, 5g/L of citric acid to ensure pH < 3.0, and an SO_2 addition of 280 mg/L) and four solutions of phenolic concentrates at doses of 4, 2, 1, and 0,5 mL/L in deionized water. The contaminated cubes were transferred to 200 mL flasks (one cube per flask) containing 100 mL of the aforementioned solutions, with three repetitions per treatment. After keeping all the treatments agitated for three days, 0.1 mL of the suspension was taken from each flask and inoculated into Petri dishes in two dilutions (10^1 and 10^2). The culture on the discs was left in the thermostat at 25 °C to grow. Three days after seeding the Petri dishes the suspension was taken from a thermostat, and the effect of different treatments was compared, counting the colonies that could survive, using the Koch's method of plate counting [38].

Before starting the experiments, all the flasks, volumetric containers, forceps, cylinders and other laboratory utensils, which could be of use, as well as the oak cubes, were also autoclaved. Sterilization was carried out with employing a Fedegari Autoklaven AG autoclave.

2.4 Statistical analysis

Statistical analyzes were performed using the statistical program R, with the R Commander package. When the populations were normally distributed and presented homogeneity in variance, parametric tests (ANOVA and Tukey) were used to detect significant differences at $p < 0.05$. Differences were considered statistically significant at $p < 0.05$. The graphs were developed with the SigmaPlot program in its version 11.

3 Results and discussions

3.1 Control of *Oenococcus oeni*

In the small-scale essay, with Chardonnay wine (250 mL cylinders), all treatments with phenolic concentrates maintained similar levels of malic acid, with small fluctuations, which can be explained by the precision range of the analysis methodology. This indicates that even at the lowest tested dose of 1 mL/L, phenolic concentrates were able to inhibit the growth of *O. oeni* at the tested inoculation dose of 10^6 CFU/mL, under given conditions. Both control treatments, including SO_2, initiated malolactic fermentation, which was demonstrated by the degradation of malic acid through the weeks in which the evaluation was carried out.

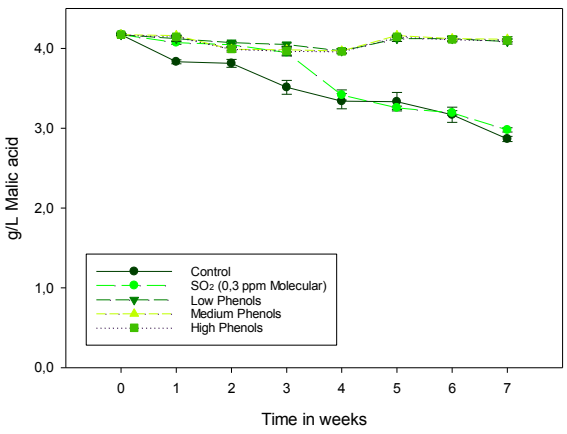

Figure 1. Concentration of malic acid in Chardonnay wine. Inhibition effect of plant phenolic concentrates on *O. oeni.*

The control treatment without additions of antimicrobial agents began to metabolize malic acid during the first week, while the treatment with SO_2 started the degradation of malic acid from the third week. The latter can be caused by the constant stirring, which induced the wine aeration and depletion of free SO_2 leading to lower protection against LAB. Figure 1 shows the evolution of malic acid by the evaluated treatments during 7 weeks of the experiment.

In larger scale trials with Riesling wines (in 50 L containers), the results confirmed the inhibitory capacity of the phenolic concentrates on the development of malolactic fermentation, compared to the control treatments (without antimicrobials agents). The levels of malic acid and lactic acid after six months storage after inoculation with *O. oeni* are shown in Table 4. It is observed that both treatments with SO_2 (20 mg/L of free SO_2) and phenolic concentrates effectively inhibited the development of malolactic fermentation, while the control treatment without additives developed MLF normally during the course of time, after the inoculations performed (degradation of malic acid to lactic acid). These results were for both Riesling wine vinified from healthy and botrytized grapes.

Table 3. General wine parameters of Riesling wines inoculated with LAB after 6 months of storage. All the values are the means ± standard deviations of three replicates. The letters represent the significant differences between the different treatments.

Riesling wines from botrytised grapes						
Treatment	**Control**	**SO_2**	**Phenols**	**C**	**SO_2**	**Phen**
Malic acid g/L	0,30 ± 0,17	4,00 ± 0,00	3,80 ± 0,06	a	b	b
Lactic acid g/L	2,50 ± 0,10	0,37 ± 0,00	0,40 ± 0,00	a	b	b
Free SO_2 mg/L	0,00 ± 0,00	17,0 ± 0,00	0,00 ± 0,00	a	b	a
Tot SO_2 mg/L	18,7 ± 0,60	109,0 ± 1,00	19,0 ± 0,00	a	b	a
Tot phenol mg/L	168 ± 0,00	242 ± 0,00	441 ± 0,00	a	b	c
Riesling wines healthy grapes						
Treatment	**Control**	**SO_2**	**Phenols**	**C**	**SO_2**	**Phen**
Malic acid g/L	0,07 ± 0,06	3,60 ± 0,00	3,40 ± 0,06	a	c	b
Lactic acid g/L	2,80 ± 0,00	0,57 ± 0,06	0,53 ± 0,06	a	b	b
Free SO_2 mg/L	2,00 ± 0,00	17,0 ± 0,00	2,00 ± 0,00	a	b	a
Tot SO_2 mg/L	12,3 ± 0,58	77,3 ± 0,58	12,3 ± 0,58	a	b	a
Tot phenol mg/L	133 ± 0,58	168 ± 0,00	314 ± 0,00	a	b	c

3.2 Control of *Brettanomyces sp.*

The development of undesirable *Brettanomyces* yeasts most often occurs when wine is in contact with wood, e.g., during the storage in oak barrels. Repetitive usage of wooden vessels can increase the risk of wine contamination by the spoilage microorganisms. Therefore, sanitizing of oak barrels, staves, etc., is of great importance. Conventional sanitizing solutions based on water with citric acid and SO_2 can be replaced in the future by more environmentally friendly and safer products. An aqueous solution of plant phenolic extracts was examined in this work as an alternative sanitizer for wooden materials.

Both sanitizing solutions were tested to treat the contaminated oak cubes with *Brettanomyces* yeasts. The solutions after the treatment of the oak cubes were placed into Petri dishes for three days and the subsequent yeast counts result are shown in Fig. 5, (the values expressed in percentage and in logarithmic reduction). The results were compared to the control (treatment with water).

In the control treatments, it was possible to establish the normal development of the colonies, allowing comparison with the different treatments tested. Regarding the efficacy of the phenolic concentrates, a clear inhibition of the development of *Brettanomyces* is observed, with different percentages of inhibition according to the doses tested. At concentrations of 4 mL/L the maximum effect was observed, completely neutralizing the yeasts with a total inhibition of their growth, which is comparable to the treatment of SO_2 with citric acid, in which the same pattern was observed for these treatments. For the other doses tested, the phenolic concentrates had an inhibitory effect that was calculated in comparison with the control treatments, proving that although they do not completely control their growth, they do have an effect on the reduction of yeast populations. Compared to the control, where the average amount of yeast was $35x10^3$ CFU/mL, the phenolic treatment dosed at 2 mL/L reduced the *Brett*. content by 96.57% (log 1,46); at 1 mL/L reduced the *Brett.* content by 78.22% (log 0,66), while 0.5 mL/L reduced the *Brett.* content by 53.56% (log 0,33). Consequently, the efficiency of the phenolics concentrates, even at the lowest doses tested, exceeds 50%.

Table 4. Amount of *Brettanomyces* cells grown (in Petri dishes) from the sanitizing solutions after their exposure to the contaminated oak cubes.

Composition of the Solution	Yeast Amount (CFU/mL)	Neutralized Yeast (%) Compared to Control	Logarithmic reduction
Distilled water (control)	34.667 ± 3.786	---	---
SO_2 + citric acid	0 ± 0	100,00	---
Phenols 4 ml/L	0 ± 0	100,00	---
Phenols 2 ml/L	1.190 ± 85	96,57	1,46
Phenols 1 ml/L	7.550 ± 3.465	78,22	0,66
Phenols 0,5 ml/L	16.100 ± 1.414	53,56	0,33

4 Conclusions

It can be concluded that the evaluated plant phenolic concentrates effectively inhibit the growth of the tested microorganism populations. Both *O. oeni* and *Brett.* showed a response to the treatments, in which the inhibition was observed both by verification of by-products of *O. oeni* metabolism (malic and lactic acids), and by counting the development of *Brett*. populations after the treatments.

The efficiency of the phenolic concentrates was comparable to that of the SO_2 treatments, implying that the phenolic concentrates could be a feasible alternative in the substitution or decrease of the use of SO_2 in winemaking. In the micro-vinification trials to control *O. oeni*, the phenolic concentrates were efficient in inhibiting the development of malolactic fermentation at all doses tested, suggesting that they might be used as an alternative strategy to control the development of malolactic fermentation if necessary. These results were confirmed in larger-scale fermentations in the

experimental cellar, where traditional vinifications treated with phenolic concentrates achieved an inhibition of bacterial development similar to SO_2 treatments, while only the control treatments without antimicrobial agents were capable of degrading malic acid, developing malolactic fermentation. During the essays with *Brett*, it was observed that all of the doses tested proportionally reduced the development populations of this micro-organism after the treatments. Total inhibition was obtained at phenolic concentrate concentrations of 4 mL/L. The findings of this study, which involved oak contamination in the transfer and survival of *Brett.* after treatments, suggest that phenolic concentrates, in addition to being used to inhibit the development of this microorganism directly in the wine, could also be used to preserve barrels and wooden containers, which are traditionally preserved with the water solution acidified with citric acid and SO_2.

The observed results, while promising, need be supplemented in other conditions, other spoilage microorganisms, and other strains of microorganisms already examined in order to confirm the given results. It also remains to be investigated how the effectiveness and mode of action will be maintained over time, offering protection to the treated wines.

We would like to heartily thank to the staff of the microbiology department of the University of Geisenheim, especially to Dr. Christian Von Wallbrunn and Dr. Niël Van Wyk for their help in performing the experiments.

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