

Article

Pre-Fermentative Addition of Sodium and Calcium Bentonites on Chardonnay Wine Changes Heat Stability, Fermentation Kinetics, Chemistry, and Volatile Composition [†]

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Abstract

Protein stabilization in white wines commonly involves bentonite fining, yet the influence of bentonite type, dosage, and pre-fermentative treatment on wine composition and fermentation remains underexplored. This study assessed the effects of pre-fermentative additions of sodium and calcium bentonites at three dosage levels (24, 48, and 72 g/hL) on the fermentation kinetics, protein (heat) stability, and chemical and aromatic composition of Chardonnay wines under commercial winemaking conditions. Sodium bentonite at 72 g/hL achieved near-complete protein stabilization (Δ NTU = 3), while all calcium bentonite treatments required significantly higher cumulative dosages (up to 216 g/hL). Pre-fermentative bentonite additions led to modest reductions in primary amino nitrogen (up to 13.2 mg/L), resulting in extended alcoholic fermentation durations by up to 33 h and variable delays in malolactic fermentation across treatments. Volatile ester analysis revealed limited sensory impact, with isoamyl acetate showing the greatest reduction (up to −2.8 odor activity value; −39%) at higher bentonite levels, whereas ethyl decanoate remained largely unaffected. Overall, the pre-fermentative addition of sodium bentonite at 72 g/hL provided an effective strategy to reduce the need for post-fermentation fining while preserving key chemical and aromatic attributes of Chardonnay wine.

Keywords: bentonite; heat stabilization; chardonnay; esters; fermentation kinetics



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1. Introduction

Hazing or turbidity in white wines is a major instability and is generally regarded as a defect that compromises consumer acceptance. Grape-derived pathogenesis-related (PR) proteins, particularly thaumatin-like proteins and chitinases, are the primary contributors to haze formation in white wines [1–3], with chitinases generally considered the main agents responsible for instability under enological conditions [4,5]. Pathogenesis-related proteins are primarily located in the pulp cells of grape berries and are synthesized in response to environmental stressors such as heat, drought, and fungal infections [4]. These proteins are introduced into the juice during grape processing, particularly during crushing, when cell structures are disrupted. In wine, PR proteins behave as unstable colloids due to their tendency to aggregate and precipitate under conditions of elevated storage temperatures (especially above 40 °C), low pH (<3.3), and high alcohol content (>13% v/v) [6]. Their

instability forms the basis of heat stability testing, which relies on the heat-sensitivity of these proteins. Standard assays measure turbidity changes (expressed in nephelometric turbidity units, NTU), before and after thermal treatment, typically involving incubation at 50 to 90 °C for 2 to 24 h [6]. A change in turbidity higher than 2 NTU is usually considered an indication that the wine is heat unstable [4].

Preventing hazing in white wines is a standard practice that is commonly addressed by adding bentonite, a natural clay, that, under wine matrix conditions, has a net negative charge that can bind and precipitate with positively charged PR proteins. The binding capacity of bentonite is tightly related to its mineral composition and layered molecular structure, which defines its cation exchange capacity, swelling ability, and net negative charge [6,7]. The net charge is typically balanced by sodium or calcium cations that are located at the interlayer spaces of the tetrahedral structure of the clay [4,8]. When hydrated, bentonites can expand (swell) and increase their surface area, ultimately enabling them to adsorb positively charged molecules such as grape proteins through electrostatic interactions, hydrogen bonding, and Van der Waals forces [9]. Commercially available bentonites for winemaking are often referred to as sodium or calcium based bentonites, depending on predominant composition of these elements, ranging from 3 to 11% and 3 to 17% respectively [8]. Sodium-based bentonites are known for having a stronger protein binding capacity and forming non-compact precipitates; conversely, calcium bentonites are known to have weaker binding capacity, while forming compact precipitates [6]. A weaker binding capacity implies that higher dosages are typically required to accomplish protein stability, and a poor settling capacity requires additional filtration steps to prevent yield losses that may range from 3 to 10% [3]. To compensate for the deficiencies from each type of bentonite, some suppliers offer blended sodium and calcium bentonites, as well as activated calcium-based bentonites with a higher sodium content.

Some of the main drawbacks from implementing fining treatments with bentonite include the non-specific binding nature and the yield loss (3 to 10%) derived from the separation processes of racking or filtration [10]. Besides unstable proteins, bentonite can remove compounds that contribute to wine quality attributes such as esters, varietal thiols, and phenolic compounds that contribute to color and mouthfeel [11–17]. Conflicting results have been found in terms of the influence on volatile compounds; some reports have found either a minimal or a positive effect on esters of Sauvignon Blanc, Malvazija Istarska, Macabeo, and Albariño wines [3,15,18], whereas others have found a significant decrease in esters when using high bentonite dosages (80 to 100 g/hL) on Chardonnay wines [14]. In addition, bentonites may increase the likelihood of developing other wine instabilities such as the formation of calcium tartrate crystals and may significantly increase the concentration of metals that participate in oxidation reactions of polyphenols in wine (notably iron) and legally controlled heavy metals [8].

Bentonite additions in white winemaking can be implemented before, during, or after alcoholic fermentation with varying degrees of stabilization and effects on wine composition. Post-fermentative additions are typically performed towards the end of the winemaking process, once the protein content of wines is not changing drastically and the biggest fraction may have already precipitated due to interaction with matrix elements (notably phenolic compounds). Post-fermentative additions allow for a more precise and single intervention, and in addition, they offer the possibility of removing contaminants with adverse consumer health effects such as biogenic amines (i.e., putrescine) produced by some bacterial strains during malolactic fermentation [19]. Bentonite additions during fermentation have been shown to enhance fermentation kinetics and minimize the impact on sensory profiles of wines [20,21], but they may require subsequent additions to guarantee protein stability. Finally, pre-fermentative additions do not regularly focus

on achieving heat stability, but instead, they aim at adjusting the must turbidity to 50 to 200 NTU as a strategy to minimize the yeast biosynthesis of higher alcohols (derived from fatty acids), volatile sulfur compounds (derived from amino acids), and browning potential from polyphenols oxidation [22,23]. Additionally, pre-fermentative additions allow for removing oxidative enzymes that can have an impact on the oxidation of phenolic compounds and volatile compounds [23]. Ultimately, pre-fermentative bentonite additions can provide a logistical advantage by skipping the final heat stabilization towards the end of the winemaking process and can avoid potential interactions between flavor active compounds that develop during fermentation and ageing. Despite these potential benefits, pre-fermentative bentonite additions have not received as much attention in the literature as fermentative and post-fermentation applications, and only a few reports have studied their capacity to accomplish heat stability [21]. The strong varietal effect on the bentonite dosage required to stabilize grape juice has been reported as approximately 60 g/hL for Riesling, 120 g/hL for Sauvignon Blanc, and over 130 g/hL for Semillon [21]. This variance of results underscores the importance of investigating its impact on Chardonnay, one of the most widely planted white grape varieties worldwide. Moreover, the known interactions with bentonite types (calcium, sodium, activated, etc.) and the conflicting results on the chemical and sensory effects [10], call for a continuous evaluation of bentonite treatments.

The goal of this work is to evaluate the effect of pre-fermentative additions of sodium- and calcium-based bentonites at different dosage levels on (1) their capacity to achieve protein heat stability, (2) their influence on alcoholic and malolactic fermentation kinetics, and (3) the resulting chemical composition of Chardonnay wines. This study was conducted at an industrial scale using commercially harvested grapes from the Central Coast of California, providing practical insight into the applicability of pre-fermentative bentonite fining under real-world winemaking conditions.

2. Materials and Methods

2.1. Grapes

Vitis vinifera Chardonnay grapes (Clone 5, Freedom rootstock, and planted in 1997) were manually harvested on 16 October 2023 from the Center of Effort estate vineyard in Edna Valley, San Luis Obispo County, California (USA). The fruit was harvested into 13 macro bins containing 0.5 t each for a total of 6.5 tons and immediately transported to the winery. Upon arrival, the fruit was whole cluster pressed in an industrial pneumatic press to obtain a press fraction of 473 L/t. After pressing, the juice was collected into a single stainless-steel tank with the temperature control jacket at 13 °C and was homogenized continuously by sparging nitrogen and dry ice pellets. During homogenization, 40 mg/L of potassium metabisulfite was added prior to distributing 242 L into stainless steel barrels with a 280 L capacity (86% fill level).

2.2. Winemaking

The experiment design considered the addition of two food grade commercial bentonites at three dosage levels, 24, 48, and 72 g/hL, in addition to a control without bentonite addition, for a total of seven treatments performed with three fermentation replicates. The two commercial bentonites differed by having a calcium- (KWK, American Colloid Co., Hoffman Estates, IL, USA) or sodium-based (Microcol™ FT, Laffort, Bordeaux, France) composition and were rehydrated and swelled according to suppliers' instructions as follows. The sodium bentonite was rehydrated using a ratio of 62 g/L water at 20 °C and swelling time of 7 h. The calcium-based bentonite was rehydrated at 100 g/L of water at 50 °C for 2 h and then swelled for 23 h at room temperature. Bentonite slurries were continuously agitated while measuring the required volumes needed for the different

dosage levels, and after addition, the wines with bentonite were manually homogenized with a stirring wand for 30 s. While each bentonite was rehydrated with different volumes of water, the maximum increase of volume corresponds to only 1% of the total must volume, and it is assumed that any differences due to dilution are negligible. After additions, all treatments were allowed to settle for 20 h at the cellar room temperature of 15 °C.

Settled must was racked from bentonite lees into clean stainless-steel barrels filled to 227 L (80% from barrel capacity). All musts were inoculated with a commercial strain of *Saccharomyces cerevisiae* yeast (EC1118™, Lallemend Oenologie, Montreal, QC, Canada), rehydrated with nutrients (Go-Ferm Sterol Flash, Lallemend Oenologie, France) following suppliers' instructions at 30 g/hL and 24 g/hL, respectively, and fermented at cellar room temperature without the support of heat exchangers. Fermentation nutrients were not added because of the high yeast assimilable nitrogen content of grapes. Fermentation was considered complete once the residual sugar content (sum of residual glucose and fructose) was below 5 g/L. At this point, vessels were topped and inoculated with malolactic bacteria *Oenococcus oeni* (Lactoenos™ B7 direct, Laffort, Bordeaux, France). After completing malolactic fermentation, wine molecular SO₂ was adjusted to 0.6 mg/L with potassium metabisulfite. Vessel headspace was sparged with nitrogen and kept at 10 °C for four weeks.

2.3. Must and Wine Basic Chemical Analysis

Must and wine analyses of pH and titrable acidity were performed using a pH Meter (Orion Star A211, Thermo Fisher Scientific, Waltham, MA, USA) and auto titrator with NaOH 0.1N (HI901W-TA, Hanna Instruments, Woonsocket, RI, USA). Acetic acid, malic acid, lactic acid, soluble solids (°Bx, glucose, fructose), yeast assimilable nitrogen (ammonia, alpha amino acids), and free sulfur dioxide were measured using an automated analyzer and corresponding enzymatic kits (Admeo SPICA, BioSystems, Barcelona, Spain). Initial must samples were taken from each stainless-steel barrel before adding bentonite. Fermentation temperature and soluble solids (measured as °Brix) were monitored daily in the morning using a digital hydrometer (DMA35, Anton Paar, Graz, Austria). Ethanol content (%vol/vol) was measured in an alcoholizer (Alcoholizer M/ME, Anton Paar, Austria). Samples for protein stability analysis were taken after the end of malolactic fermentation and analyzed by a certified external laboratory (ETS Laboratories, St. Helena, CA, USA) measuring the difference in turbidity (in nephelometric turbidity units, NTU) after heating samples at 80 °C for 2 h.

2.4. Spectrophotometric Analysis

Chromatic parameters of wine samples were analyzed using a UV-vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, CA, USA) using CIELab coordinates including L* (lightness), C* (saturation), H* (hue angle), a* (green/red component), and b* (blue/yellow component), which were calculated using the Cary WinUV color software (version 6.0, Startek Technology, Boronia, Victoria, Australia) under a D65 illuminant [24]. Chromatic differences between treatments were evaluated using the CIELab color difference (ΔE^*), corresponding to the Euclidean distance between two points (r and s) in the three-dimensional CIELab space as follows: $\Delta E^*_{r,s} = [(\Delta L^*_{r,s})^2 + (\Delta a^*_{r,s})^2 + (\Delta b^*_{r,s})^2]^{0.5}$, as previously described by [25]. A ΔE^* value ≥ 3.0 indicates that two colors are distinguishably different from each other to the naked human eye, as reported by Casassa, et al. [26]. In addition, the value of absorbance at 420 nm was measured using quartz cuvettes with a 1 mm path length.

2.5. Volatile Analysis

Volatile compound analysis was conducted following the methodology from Cebrián-Tarancón, et al. [27] with minor modifications. Wine samples were first centrifuged at

4500 rpm for 10 min, and an aliquot of 10 mL was transferred into a 10 mL screwcap glass vial together with 0.05 mL of internal standard solution (γ -hexalactone, 0.1 mL/10 mL ethanol). Stir bar sorptive extraction of volatiles was carried out by introducing a 10 mm Twister[®] stir bar with 0.5 mm film thickness of polydimethyl syloxane (PDMS, Gerstel, Mülheim an der Ruhr, Germany) and stirring at 1000 rpm for 1 h at room temperature. Volatiles were desorbed from the PDMS stir bars using helium as a carrier gas with a flow rate of 75 mL/min. The automated thermal desorption unit (TDU2, Gerstel, Germany) was programmed to heat the stir bars from 40 °C to 295 °C at a rate of 60 °C/min (total 5 min), operating in the splitless desorption mode. Desorbed volatiles were cryo-focused at −40 °C using liquid nitrogen and a cooled injection system (CIS-4, Gerstel, Germany) coupled with a liner packed with 20 mg of Tenax TA[®] (SKC Ltd., Dorset, UK). Next, the CIS was heated to 260 °C at a rate of 12 °C/min to inject the analytes into the chromatographic column. The CIS operated in the PTV solvent vent mode with a purge flow to split vent of 80 mL/min, vent of 75 mL/min, and pressure of 20.85 psi.

The desorbed volatile fraction was separated in an Agilent 8890 gas chromatograph system (GC) coupled to a triple quadrupole (QqQ) Agilent 7000D mass spectrometer (MSD, Agilent Technologies, Santa Clara, CA, USA), operating in simple quadrupole (Q). Chromatographic conditions followed the methodology from Marín-San Román, et al. [28], with minor modifications as follows: the GC oven temperature was programmed to start at 40 °C (held for 2 min), raised to 80 °C (5 °C/min, held for 2 min), raised to 130 °C (10 °C/min, held for 5 min), raised to 150 °C (5 °C/min, held for 5 min), and then raised to 230 °C (10 °C/min, held for 5 min). The MSD was operated in scan acquisition (27–300 m/z) with an ionization energy of 70 eV. The temperature of the MS transfer line was maintained at 230 °C. Volatile compound quantification was performed according to calibration curves obtained using pure standards diluted into synthetic wine (13.5% ethanol, 5 g/L tartaric acid, pH 3.6). Compound identification was performed using the NIST library of mass spectra (National Institute of Standard and Technology, Gaithersburg, MD, USA), and quantification was performed by using the ratio between the extracted ion chromatogram (EIC) GC peak area of each compound divided by the area of the internal standard γ -hexalactone. The EIC target ions (m/z), standard supplier, and purity were as follows: isoamyl acetate (70), ethyl hexanoate (88), ethyl decanoate (88), and 2-phenylethylacetate (104). All calibration standards used a purity higher than 95%, and calibration curves had an R^2 value higher than 0.98.

2.6. Statistical Analysis

The effect of bentonite treatment on must and wine composition was evaluated by one-way analysis of variance (ANOVA) using IBM-SPSS Statistics (Version 29, IBM Corp. Armonk, NY, USA). Tukey's HSD post hoc test was used to identify differences among treatments. Results correspond to the mean value of three replicates, and significant differences are reported when the p -value of the ANOVA and post hoc tests is <0.05 .

3. Results

Results are presented using abbreviations of the six treatments by combining the use of either sodium- (Na) or calcium-based (Ca) bentonites followed by their respective dosage levels at 24, 48, and 72 g/hL.

3.1. Nitrogen Composition of Juice Before Fermentation

The effect of bentonite type and dosage on the nitrogen composition of must before fermentation was mainly noticed by a decrease in the primary amino nitrogen (PAN) (Table 1). The biggest loss of PAN was observed in the sodium bentonite with a dosage

of 72 g/hL (Na72) corresponding to 13.2 mg/L (nearly 5%, $p < 0.001$) with respect to the must before bentonite. Overall ammonia concentration did not change due to bentonite treatments, except for Ca24, where the post-bentonite must had an increase of 13.5 mg/L (11%, $p = 0.007$). The lowest concentration of yeast assimilable nitrogen (YAN) was observed in Na72, representing a loss of 14.75 mg/L (4%, $p = 0.001$), with all other treatments having no significant differences. These minor losses indicate that bentonite type and dosage do not have a drastic impact on the nitrogen content of must.

Table 1. Nitrogen compound composition of Chardonnay must before and after treatment with sodium- (Na) and calcium-based bentonites (Ca) at different dosages (g/hL). Primary amino nitrogen (PAN) and yeast assimilable nitrogen (YAN) data are the mean of three replicates. Results of the Tukey HSD test for post hoc significant differences between treatments are indicated with letters for each column.

Treatment	Sampling	Ammonia (mg/L)			PAN (mg/L)			YAN (mg/L)		
Control	Pre-bentonite	123.60	± 4.93	a, b	279.60	± 0.89	a, b, c	381.20	± 4.76	a
	Post-bentonite	130.80	± 7.66	a, b	275.60	± 2.88	c, d, e	382.60	± 6.43	a
Na24	Pre-bentonite	128.25	± 6.65	a, b	281.75	± 1.26	a, b	386.75	± 4.86	a
	Post-bentonite	130.25	± 2.87	a, b	272.50	± 1.91	d, e, f	379.50	± 1.29	a, b
Na48	Pre-bentonite	122.50	± 5.00	a, b	280.25	± 2.99	a, b, c	380.75	± 2.75	a
	Post-bentonite	134.50	± 4.04	a, b	266.75	± 0.96	g, h	377.00	± 3.16	a, b
Na72	Pre-bentonite	127.25	± 6.70	a, b	277.75	± 2.22	b, c, d	382.25	± 6.65	a
	Post-bentonite	125.25	± 5.62	a, b	264.50	± 1.00	h	367.50	± 5.32	b
Ca24	Pre-bentonite	121.25	± 3.40	b	280.75	± 2.06	a, b, c	380.00	± 2.16	a, b
	Post-bentonite	134.75	± 7.54	a	271.00	± 2.45	e, f, g	381.75	± 7.27	a
Ca48	Pre-bentonite	125.50	± 2.38	a, b	280.50	± 2.89	a, b, c	383.50	± 3.32	a
	Post-bentonite	130.75	± 1.26	a, b	269.25	± 2.50	f, g, h	376.25	± 3.10	a, b
Ca72	Pre-bentonite	126.00	± 6.06	a, b	283.25	± 3.40	a	387.00	± 8.12	a
	Post-bentonite	133.50	± 6.35	a, b	270.00	± 1.15	f, g	379.75	± 5.56	a, b
ANOVA p -value		0.007			<0.001			0.001		

3.2. Alcoholic Fermentation Kinetics

Alcoholic fermentation followed a normal development in all treatments, and the most notable difference was related to the time to reach 0 °Bx (Figure 1). The lower dosage bentonite treatments (Ca 24 and Na24) took 9.6 and 19.2 h longer than the control to reach 0°Bx, while the higher dosage treatments for both bentonites took, on average, 33.6 h longer with respect to the control. The longer fermentation time could be potentially related to the small differences in YAN noticed in Table 1. All treatments reached a −1.0 Bx after 9 ± 0.4 days of fermentation. At this point, wines were racked into new stainless-steel kegs and inoculated with lactic acid bacteria.

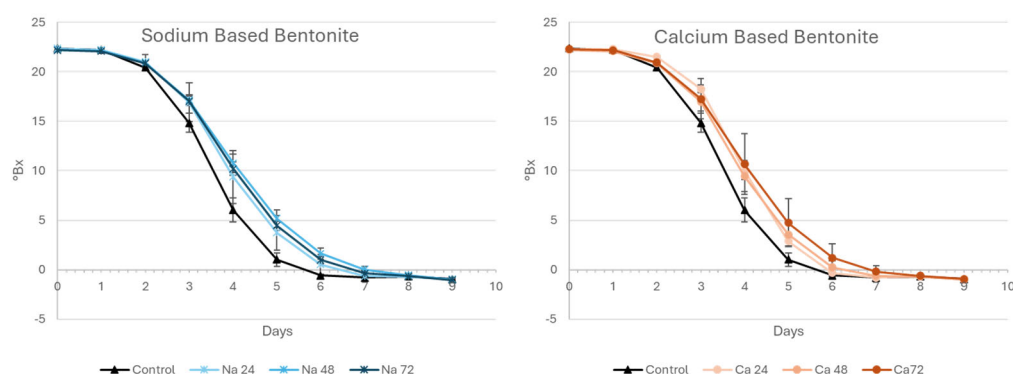


Figure 1. Fermentation curves of control and bentonite treatments by dosage rate (in g/hL). Error bars represent standard deviation of 3 replicates.

Fermentation temperature for all treatments ranged between 16 and 22.4 °C, with an average maximum of 21.3 °C between day 4 and 5. All treatments had a similar temperature profile, except for Ca24, which reached 22.4 on day 5 corresponding to a 1 °C difference with respect to all other treatments.

3.3. Malolactic Fermentation Development

Malolactic fermentation kinetics were slower in the bentonite treatments compared to the control, as shown Table 2. The control vessels were the first to reach a concentration below 0.1 g/L after one month from inoculation followed by the Na48 treatment, whereas the rest of the bentonite treatments took approximately 2 months to reach a concentration below 0.1 g/L of malic acid.

Table 2. Malic acid concentration (in g/L $\pm$ standard deviation) of the sodium (Na) and calcium (Ca) bentonite treatments at different dosage rates (g/hL) after inoculation of lactic acid bacteria. Data are means of 3 replicates. *p*-values from ANOVA in the last row indicate significant differences if *p* < 0.05.

Treatment	February			March			April		
Control	3.87	$\pm$	0.19	0.03	$\pm$	0.02	0.06	$\pm$	0.01
Na 24	2.91	$\pm$	2.13	0.68	$\pm$	1.16	0.05	$\pm$	0.01
Na 48	3.30	$\pm$	1.30	0.02	$\pm$	0.03	0.04	$\pm$	0.01
Na 72	4.03	$\pm$	0.48	0.65	$\pm$	1.07	0.06	$\pm$	0.02
Ca 24	3.31	$\pm$	1.00	0.26	$\pm$	0.40	0.05	$\pm$	0.01
Ca 48	4.31	$\pm$	0.06	1.14	$\pm$	1.48	0.06	$\pm$	0.01
Ca 72	4.20	$\pm$	0.08	0.97	$\pm$	1.65	0.04	$\pm$	0.01
<i>p</i> -value	0.668			0.778			0.222		

The effect of bentonite dosage on malolactic fermentation was mostly noticed with the calcium bentonite, where higher concentrations lead to slower metabolism and a higher variance across replicates (Table 2). Regardless of the treatment, it was surprising to observe a variation of up to 1.65 g/L within replicates of a single treatment (such as Ca72, *n* = 3). While no significant differences were observed due to the large variance, Ca48 was left with the highest leftover malic acid after one month, and it is possible to observe that bentonite treatments in general slowed down malolactic fermentation.

3.4. Heat Stabilization

After the end of malolactic fermentation, wines were submitted to a heat stability test that demonstrated that the sodium bentonite treatment removed a significantly larger fraction of unstable proteins in comparison to the control and calcium treatments (Figure 2, *p* < 0.05). Increasing sodium bentonite dosage removed a higher fraction of unstable proteins, with Na72 being the only treatment that produced a Δ NTU value of 3 and, therefore, allowing to consider the wine as very close heat stability (Δ NTU < 2). Turbidity (NTU) decreased linearly with increasing sodium bentonite dosage, with each increment resulting in an approximate 10 NTU reduction. In contrast, calcium bentonite led to a noticeable decrease in turbidity only at the highest dosage level (72 g/hL), and even then, its effect was comparable to that of the lowest sodium bentonite treatment (24 g/hL). The intermediate and high dosage treatments of sodium bentonite (Na48, Na72) produced a more consistent Δ NTU compared to Na24 and all the calcium bentonite treatments, which highlights the efficacy of these treatments, possibly due to better interaction with the unstable proteins during the pre-fermentative period.

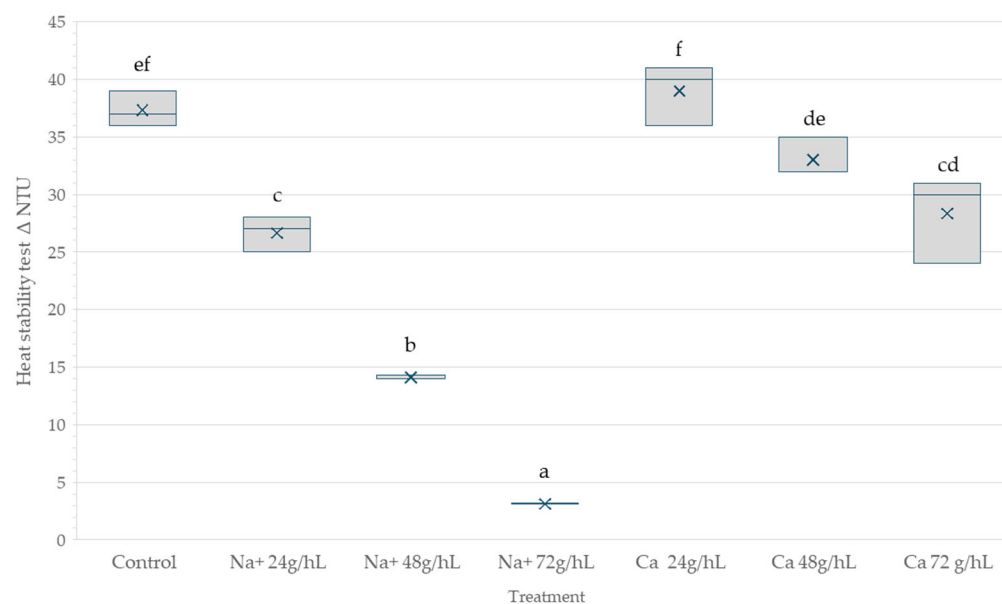


Figure 2. Heat stability test of the control and bentonite-treated wines ($n = 3$) following the completion of malolactic fermentation. A difference in nephelometric turbidity units (NTU) of less than 2 is generally considered indicative of heat stability. Box sizes represent the 95% confidence interval from three replicates; means are marked with an “X” and medians by a horizontal line within each box. Different letters above the boxes indicate statistically significant differences between treatments ($p < 0.05$), as determined by Tukey’s HSD post hoc test.

Results from Figure 2 required that all wines were subject to a final bentonite addition as part of the standard procedure in the commercial winery where the experiment was conducted. For this purpose, amounts of 48, 24, and 12 g/hL of the same sodium bentonite were added to the Na24, Na48, and Na72 treatments for a total addition of 72, 72, and 84 g/L, respectively. These results highlight that the lower dosages required the same amount of total bentonite to accomplish stability (72 g/hL) as the highest dosage treatment, which by itself was only short of 1 NTU to be considered stable. In contrast, the calcium treatments required additional amounts of 168, 150, and 144 g/hL, which accounted for a total addition of 192, 198, and 216 g/hL to the Ca24, Ca48, and Ca72, respectively. These results clearly demonstrated the superior performance of the sodium-based bentonite and the possibility to accomplish a more consistent pre-fermentative protein stabilization of the must at a dosage rate that falls within common practice.

3.5. Chemical and Visual Parameters

The chemical composition of the wines following heat stabilization was largely unaffected by bentonite addition, regardless of dosage level (Table 3). A small but statistically significant reduction in alcohol content was observed across treatments, ranging from 0.10% to 0.27% *v/v* ($p < 0.001$). In addition, the calcium bentonite treatments had significantly lower alcohol content compared to the sodium treatments, regardless of dosage rate ($p < 0.001$). While the opposite could be expected due to the higher swelling capacity in sodium bentonites and, therefore, a higher volume of alcohol adsorbed in the interlayer space, this aspect may deserve further research. Albeit significant, these small differences in alcohol content are unlikely to be of sensory relevance for consumers, as perceivable differences in alcohol content in Chardonnay wines have been reported to range from 0.5 to ~1.5% [29,30], that is, well above the difference range herein observed. Nonetheless, minor effects of differential volatility of certain aromas could be surmised even at low ethanol ranges, or even minor shifts in the perception of wine’s structural elements [31]. However, the present work did not evaluate the sensory composition of the wines.

Table 3. Effect of bentonite treatments on chemical and chromatic parameters of Chardonnay wines after final heat stabilization. Mean values from three replicates with $\pm$ standard deviation are shown together with p -values from ANOVA. Abbreviation for residual sugars glucose and fructose (Glu + Fru) and titrable acidity (TA).

Treatment	Glu + Fru (g/L)				Alcohol%			TA (g/L)			pH		Acetic Acid (g/L)				
Control	0.82	±	0.04	a, b, c	13.26	±	0.02	a	8.47	±	0.39	3.60	±	0.02	0.31	±	0.03
Na 24 g/hL	0.88	±	0.06	a, b	13.16	±	0.03	b	8.11	±	0.41	3.64	±	0.04	0.29	±	0.01
Na 48 g/hL	0.91	±	0.02	a	13.20	±	0.03	a, b	8.14	±	0.22	3.61	±	0.01	0.27	±	0.04
Na 72 g/hL	0.73	±	0.06	c	13.14	±	0.02	b	8.34	±	0.07	3.60	±	0.02	0.26	±	0.06
Ca 24 g/hL	0.81	±	0.03	a, b, c	12.99	±	0.03	d	8.30	±	0.11	3.61	±	0.01	0.31	±	0.01
Ca 48 g/hL	0.78	±	0.06	b, c	13.00	±	0.02	c, d	8.28	±	0.19	3.61	±	0.01	0.25	±	0.02
Ca 72 g/hL	0.81	±	0.04	a, b, c	13.06	±	0.03	c	8.49	±	0.09	3.61	±	0.01	0.24	±	0.02
<i>p</i> -value	0.005				<0.001			0.433			0.210		0.075				

No significant differences in residual sugar were observed between the control and bentonite treatments; however, Na48 exhibited the highest concentration (0.91 g/L), being 0.18 g/L higher than Na72 and 0.13 g/L higher than Ca48 ($p = 0.005$). The acid profile—comprising pH, titratable acidity (TA), and acetic acid—remained unchanged across all treatments ($p = 0.075$). Additionally, no significant differences were found in absorbance at 420 nm, CIELAB color parameters, or ΔE^* , indicating that the bentonite addition had no measurable impact on the visual characteristics of the wines (see Supplementary Materials Table S2).

3.6. Volatile Esters Composition

The concentrations of four volatile esters were evaluated following malolactic fermentation and subsequent heat stabilization to assess potential aroma modifications induced by the different treatments. Results are expressed as odor activity values (OAVs), calculated as the ratio of the compound's concentration in the sample to its odor threshold as reported in the literature.

Following the completion of malolactic fermentation (Figure 3a, Table S1), the effects of pre-fermentative bentonite dosage were most evident at the highest addition rates for both sodium and calcium bentonites. The Na72 treatment resulted in reductions of 9 OAV units in isoamyl acetate ($p < 0.001$, -39%) and 4.4 OAV units in ethyl decanoate (-7%) compared to the control. Similarly, the Ca72 treatment caused losses of 6.5 OAV units in isoamyl acetate ($p < 0.001$, -28%) and 17 OAV units in ethyl decanoate ($p = 0.01$, -28%). Although a downward trend in total ester concentration was observed with increasing sodium and calcium bentonite dosage, this pattern was not statistically conclusive, and Ca72 was the only treatment that showed a clear loss in total esters of 70 OAV units with respect to the control ($p = 0.001$). The loss of isoamyl acetate in both bentonite treatments is in agreement with results on base wine for Chardonnay sparkling wine [16]; nevertheless, we only observed a decrease of ethyl hexanoate in Ca24 ($p = 0.014$), which did not clearly define a dosage effect.

After the final heat stabilization (Figure 3b, Table S1 [32]), both the type and dosage of bentonite influenced the concentrations of specific volatile esters, although no consistent dose-dependent patterns were observed across treatments. All bentonite-treated wines exhibited a decrease in isoamyl acetate ($p < 0.001$), with losses ranging from 1.1 to 2.8 OAV units relative to the control, and higher losses observed with higher dosages for both sodium and calcium treatments. Significant differences were also detected for ethyl hexanoate ($p = 0.007$), and 2-phenylethyl acetate ($p < 0.001$); however, the magnitude of these changes

remained below 1 OAV unit, suggesting limited sensory impact. Although overall trends were not strongly correlated with dosage, the Na24 treatment (the lowest sodium bentonite level) resulted in the smallest decrease in isoamyl acetate and the largest increase in ethyl decanoate, pointing to a potential selective or protective effect at lower sodium bentonite concentrations (Figure 3b). Because ethyl decanoate was the molecule with the largest OAV value, the absence of significant differences among the treatments suggests that bentonite additions had a discrete effect on the fruity character of the wines.

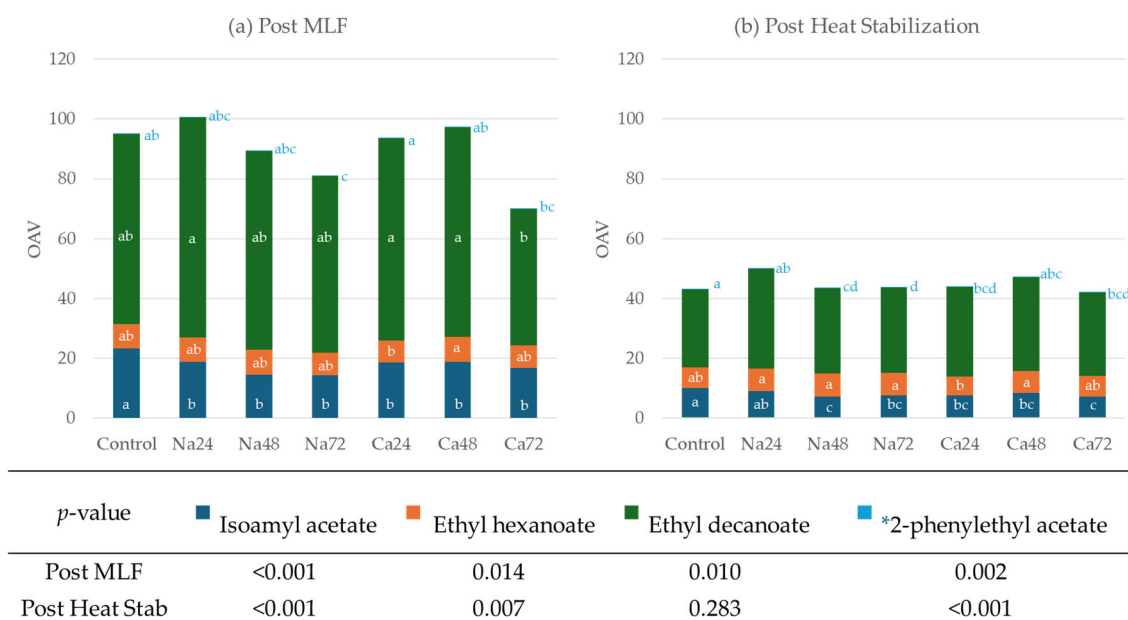


Figure 3. Odor activity values (OAVs) of volatile esters in Chardonnay wines subjected to pre-fermentative bentonite treatments (Na sodium and Ca calcium bentonite with dosages in g/hL), measured at (a) the end of malolactic fermentation (MLF) and (b) after the final heat stabilization step. For each compound, different letters inside the colored bars indicate statistically significant differences between treatments ($n = 3$), as determined by ANOVA and by Tukey's post hoc test. The ANOVA p -values for each volatile are displayed below the legend. *2-phenylethyl acetate post hoc group letters are indicated next to the bar from each treatment due to their small value; readers are referred to the Supplementary Materials to see concentration and OAV values for this compound.

A comparison of ester profiles between the end of malolactic fermentation and after the final heat stabilization (involving a second bentonite addition) revealed pronounced losses in all esters evaluated. Ethyl decanoate experienced the most substantial decline (−34 OAV units, −54%), followed by isoamyl acetate (−9.7 OAV, −53%), ethyl hexanoate (−0.8 OAV, −10%), and 2-phenylethyl acetate (−0.02 OAV, −41%). On average, heat stabilization led to a reduction of approximately 45 OAV units, equivalent to a 50% loss of the total ester content. Collectively, these findings underscore the vulnerability of aroma-active esters to post-fermentative processing and highlight that individual compounds differ in their susceptibility to loss.

4. Discussion

Achieving early heat stability is one of the main purposes of pre-fermentative bentonite additions, and our results indicate that only the sodium-based bentonite at a concentration close to 72 g/hL would be able to accomplish this objective. This result is in agreement with previous research and is explained by the superior swelling capacity, higher negatively-charged surface, and adsorptive capacities per unit of agent in the platelets of sodium bentonites [4,33]. While this might be case-specific and thus true to the local Chardonnay

grapes of the present study, winemakers should perform bench trials that provide a more accurate representation of the protein composition of their grapes and pre-fermentative must composition.

Our results showed that the pre-fermentative addition of sodium and calcium bentonite at different concentrations on Chardonnay juice produced an average loss of 4% of primary amino nitrogen (Table 1). Sodium-based bentonite produced an almost linear decrease in primary amino nitrogen, with a loss of up to 13 mg/L at the highest dosage rate (72 g/hL), while this trend was not observed for the calcium-based bentonite. These results contrast with those found by Weiss and Bisson [20], who reported that pre-fermentative bentonite additions had no effect on the nitrogen composition of Chardonnay musts regardless of dosage rate. The fact that we used the same brand of sodium bentonite, similar dosage rates, and similar must pH underscores how other variables such as grape origin might play an important role in the interaction between must composition and bentonite. It is possible that our must contained a higher proportion of amino acids that had a higher affinity for the bentonites used, as shown for different PR proteins [34].

While changes in the primary amino acids and yeast assimilable nitrogen of the wines may seem minor, they could be related to the slowdown in the alcoholic fermentation observed in our results. All bentonite treatments took an average of 26 additional hours to reach 0 °Bx in comparison with the control, with the highest dosages (48 and 72 g/hL) producing a delay of 32 h regardless of bentonite type. A similar slowdown in fermentation time as a function of pre-fermentative bentonite fining has been observed in reports with Chardonnay wines but not in Sauvignon Blanc wines [20]. Weiss and Bisson [20] also observed a decrease in fermentation rate as a function of bentonite addition, which was slightly accentuated by increasing bentonite dosage rate. Our results show a similar trend where all bentonite treatments had a lower maximum fermentation rate compared to the control, and the highest decrease was observed with the 48 and 72 g/hL dosages regardless of bentonite type. The minor differences in fermentation temperature among treatments suggest that temperature was not responsible for the ~26 h delay in reaching dryness. Instead, this slowdown is likely related to the removal of nutrients by bentonite, as previous studies have shown that bentonite can adsorb not only proteins but also other micronutrients important for yeast metabolism, such as fatty acids, and sterols, which may impair yeast growth and fermentation kinetics [14,35]. For example, Lambri et al. [14] showed that bentonite removed hexanoic acid and octanoic acid when applied at dosages between 50 and 100 g/hL. Moreover, Cocito and Delfini reported that pre-fermentative clarification treatments including cold static clarification combined with the addition of 10 g/L of gelatine and 40 g/L of bentonite caused the strongest loss of long chain fatty acids (C14 to C18) and sterols (including grape derived β -sitosterol and 5- α -cholestane, and yeast derived ergosterol, lanosterol, and squalene) compared to cold *débourbage* without clarifier agents and to flotation [35]. Because sterols are known to be responsible for the maintenance of yeast cell viability and to prevent sluggish and stuck fermentations [36], it is likely that the bentonite treatments removed a significant fraction and caused the observed delay in fermentation time [20]. Beyond the performance on fermentation kinetics, both sterols and fatty acids have also been shown to modulate the composition of esters and higher alcohols produced by yeast during fermentation [36]. While the 26 h delay to reach 0 °Bx may seem discrete, this time can make a difference in the logistics of medium- and large-scale wineries, where tank turnover plays an important role in processing capacity and efficiency.

Despite malolactic fermentation being widely practiced in Chardonnay wine production, reports linking delays of malolactic fermentation to bentonite addition are scarce mainly because the scope for most white wine research does not include this secondary

fermentation as a standard protocol. Our results highlight that bentonite addition, regardless of bentonite type and dosage, created a substantial variability in the amount of malic acid metabolized by the lactic acid bacteria inoculated (*Oenococcus oeni*). In many treatment replicates, we observed a delay of nearly one month to complete malolactic fermentation with respect to the control. This delay may stem from the removal of micronutrients that are important for lactic acid bacteria, such as fatty acids (notably decanoic, dodecanoic, and oleic acid) or amino acids (notably arginine, glutamic acid, tryptophan, and isoleucine) [37]. A trend suggesting a higher inhibition and variance with an increase in dosage level was only observed for the calcium bentonite one month after inoculating the bacteria. Because our experiment only involved inoculating a single commercial strain of *Oenococcus oeni*, other strains may have a different effect. This finding calls for more research on the potential inhibitory effect of bentonite on lactic acid bacteria.

In the finished wines, pre-fermentative additions of bentonite produced an average loss of 2 OAVs from isoamyl acetate and an average gain of 4 OAVs in ethyl decanoate, which indicates that these minor changes may compensate each other and render wines with similar fruity intensities. Dosage or bentonite type effects were not conclusively observed in the finished wines, and our findings align with other studies that also found only minor differences in the volatile composition when using pre-fermentative bentonite additions [18]. A study on Malvazija Istarska white wines showed that bentonite additions added at the end of alcoholic fermentation produced wines with higher ester concentrations compared to the control, potentially due to a decrease in turbidity, and that the resulting wines had a higher fruity aroma when analyzed by descriptive sensory analysis [3]. Increases in esters have also been found in Sauvignon Blanc wines with sodium-activated bentonite additions during fermentation [15] and in Chardonnay sparkling wines with bentonite additions at tirage [16]. In contrast, post-fermentative additions of calcium-based bentonite to Chardonnay wines have been reported to produce a dose dependent loss of ethyl hexanoate, ethyl octanoate, and isoamyl acetate with losses up to 5, 40, and 60%, respectively [14]. It is likely that these drastic differences with our results stem from the post-fermentative bentonite addition used, the higher dosage rate (100 g/hL), and the notably smaller wine volume used in the experiment (4 L demijohns).

Overall, the lack of significant differences of the total esters between the control and treatments in our last sampling indicates that pre-fermentative bentonite treatments have only a minor effect on the fruity attributes of the wine compared to the untreated control. Nevertheless, expanding the volatile analysis to other esters, terpenes, and varietal thiols should allow us to have a better understanding of pre-fermentative bentonite additions on the volatile composition. For example, future research could corroborate if pre-fermentative bentonite treatments have the same effect as end-of-fermentation additions where an increase of terpenes like linalool, geraniol, citronellol, and nerol has been observed [3].

5. Conclusions

Pre-fermentative protein stabilization of Chardonnay wines was partially achieved with a sodium bentonite dosage of 72 g/hL and a minimal effect on the chemical and volatile composition. On the other hand, calcium-based bentonites required a significantly higher addition rate to accomplish protein stabilization, without a major impact on the composition of the wines.

The observed loss in primary amino acids before fermentation, together with the probable removal of other essential fermentation nutrients such as sterols and fatty acids, are likely contributing factors to the slowdown in both alcoholic and malolactic fermentation kinetics. The pronounced variability and extended time required to complete malolactic

fermentation in bentonite-treated wines further highlight the need for future research aimed at elucidating the potential interference of bentonite with lactic acid bacteria metabolism.

While our study did not include sensory evaluation, the minor differences observed on the concentration of major volatile esters and chemical composition suggest that consumers would be unlikely to perceive differences between bentonite-treated wines and the control, consistent with previous reports. Future studies should extend the number of volatile compounds and chemical families to provide a more comprehensive understanding of the effect of bentonite types and dosage.

The wide diversity of commercially available bentonites, including sodium, calcium, activated, and granulated products, has shown conflicting outcomes in the literature that, in addition to the diversity of grape varieties and, notably, their diverse protein contents and chemical composition, call for winemakers to constantly evaluate the performance of local products through bench trials.

The timing of bentonite addition, before, during, or after fermentation, remains a variable of interest that plays an important role in the final composition and production logistics of white wines. Our results suggest that pre-fermentative additions can offer a way to minimize post-fermentative additions and therefore minimize wine losses and chemical changes in the finished product.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation11090535/s1>, Table S1. Volatile ester concentration by type of bentonite (Na = sodium and Ca = calcium based) and dosage (in g/hL) treatments; Table S2. Effect of pre-fermentative bentonite (Na = sodium and Ca = calcium based) and dosage (in g/hL) treatments on spectrophotometric (absorbance at 420 nm) and chromatic parameters (CIELab coordinates) of Chardonnay wines after final heat stabilization.

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Abbreviations

The following abbreviations are used in this manuscript:

Na24	Sodium bentonite treatment with 24 g/hL dosage
Na48	Sodium bentonite treatment with 48 g/hL dosage
Na72	Sodium bentonite treatment with 72 g/hL dosage
Ca24	Calcium bentonite treatment with 24 g/hL dosage
Ca48	Calcium bentonite treatment with 48 g/hL dosage
Ca72	Calcium bentonite treatment with 72 g/hL dosage
NTU	Nephelometric turbidity units
OAV	Odor activity value
ANOVA	Analysis of variance

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