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Antimicrobial Activity of Chitosan from Different Sources Against Non-*Saccharomyces* Wine Yeasts as a Tool for Producing Low-Sulphite Wine

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Abstract: Chitosan is used as an antimicrobial agent in different agri-food applications; in wine-making, the use of chitosan from *Aspergillus niger* is authorized, but other sources of chitin, and consequently of chitosan, are available, such as crustaceans and insects. This work investigates the antimicrobial efficiency of chitosan from crustaceans and insects (*Hermetia illucens*) against non-*Saccharomyces* yeasts in wine. For this aim, the first step was to evaluate the effect of crustacean chitosan, tested both alone and in combination with low sulphur dioxide (SO_2) concentrations, on the cell viability of 20 non-*Saccharomyces* strains in the first step of fermentations inoculated with each strain. Furthermore, the strain resistance to crustacean- and insect-based chitosan was evaluated in agarized media, together with the addition of different antimicrobial concentrations. Finally, the efficiency of different antimicrobial treatments was evaluated during laboratory-scale fermentations inoculated with a selected *S. cerevisiae* strain. The tested strains exhibited medium/high resistance to the chitosan; in some cases, the behaviour varied in the function of species/strain, and only four strains exhibited different resistance levels, depending on the chitosan source. The addition of chitosan alone during fermentation inoculated with *S. cerevisiae* showed lower antimicrobial activity than SO_2, but the combined use with SO_2 showed a better effect than chitosan alone. The evaluation of the suitability of chitosan obtained from a sustainable source, such as insects, will allow us to give new information on the future applications of this natural compound for the production of wine with low sulphite content.

Keywords: chitosan; non-*Saccharomyces* yeasts; sulphur dioxide wine fermentation; cell viability

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1. Introduction

In wine production, microbiological control of the fermentation process is essential to promote the dominance of microorganisms of oenological interest and to inhibit, on the other hand, the development of undesirable microorganisms, such as yeasts, lactic acid bacteria, and acetic bacteria. Inadequate control can lead to irreversible effects on wine quality and considerable economic losses [1].

Thanks to its large spectrum of action, which also includes the prevention of oxidative phenomena, sulphur dioxide (SO_2) has always been the most widely used antimicrobial compound [2].

However, despite the numerous advantages offered by the use of SO_2, drawbacks related to wine quality and human health have increased the market demand for the production of wines with reduced sulphite content. Indeed, wine quality is compromised by the appearance of sensory defects, unpleasant aromas, and bad smells due to excessive doses of sulphites that are degraded by yeasts, especially in nutrient-poor grape musts, with the production of hydrogen sulphide and mercaptans [3].

With regard to the problems related to human health, adverse reactions in the gastrointestinal tract, on the skin, and in the respiratory system can occur in the "sulphite-sensitive" population after sulphite ingestion [4–6]. For these reasons, considering the large use of SO_2 in different food products, the European Union (EU) established a limit for the dosage of this compound in foods, as the risk is correlated with excessive cumulative ingestion, and the World Health Organization (WHO) estimated the allowable daily intake to be about 0.7 mg per kg of body weight [7].

In winemaking, limits on the use of this antimicrobial have also been established. Indeed, the maximum allowed dose of total SO_2 content in red wine is 150 mg/L, and in white and rosé wine, it is 200 mg/L (EU Regulation No. 606/2009 and No. 479/2008).

On the basis of these considerations, consumer attention to buying healthier products is growing, and research is focusing on the study of compounds that can replace or reduce the use of this chemical additive. For this purpose, several alternative additives have been proposed and authorized by the International Organization of Vine and Wine (OIV) [8,9]. As reported by Castro Marin et al. [10] and Lárez Velásquez [11], chitosan has shown great importance in the oenology industry for its use as a potential food preservative of natural origin.

Chitosan is a polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine, held together by β-(1-4) bonds, obtained from the deacetylation processing of chitin, a biopolymer of N-acetyl-glucosamine, the most abundant polysaccharide in nature after cellulose, and present mainly in molluscs, crustaceans, fungi, and insects [12]. As chitin is not soluble, its conversion into chitosan via the removal of acetyl groups allows us to obtain a more soluble and suitable compound for several applications in the food sector [13,14].

The OIV [15] has authorized the use of chitosan in wine at different doses for various purposes, such as to reduce the concentration of heavy metals (Fe, Pb, Cd, Cu), to prevent hazing, to reduce contamination by ochratoxin A, and to reduce the concentration of unwanted microorganisms, especially *Brettanomyces* spp.

In addition to the already authorized purposes, several studies also showed other applications of chitosan, for example, as a compound with antioxidant activity in wine [16].

The OIV has authorized the use of chitosan derived from the fungus *Aspergillus niger*, but other sources of chitin, and consequently of chitosan, are available, such as crustaceans and insects. Crustaceans also constitute an abundant source of polysaccharides, since their exoskeleton is made up of 15 to 40% chitin [17], but the use of chitin and chitosan from this source is not allowed in winemaking because of the potential release of fish protein into the product, with the potential risk of allergic reactions [18]. Regarding insect-based chitosan, to date, the U.S. Food and Drug Administration (US FDA) does not include insects as a major food allergen [19]. Insects can be considered an alternative and sustainable source of chitosan, with numerous advantages, such as the reproducibility of insect-rearing conditions that allow us to obtain chitin during the whole year [20].

Some insects also have the advantage of bioconverting waste products, as they can feed on different organic substrates [21]. Among these, great interest is being paid to the dipteran *Hermetia illucens* [22]. Chitosan obtained through the deacetylation of chitin extracted from different biomasses of *H. illucens* is characterized by a low molecular weight (MW) and a high degree of deacetylation (DD) [14].

Currently, chitosan is used in oenology mainly to control the wine-contaminating yeast *Brettanomyces bruxellensis*. Indeed, several studies have demonstrated the antimicrobial activity of chitosan against this yeast at low doses of the compound (about 40 mg/L) [23–28]. However, some studies have also shown encouraging results regarding the potential activity

of chitosan against non-*Saccharomyces* yeasts [29,30] and against both lactic acid and acetic acid bacteria, but further studies are needed to demonstrate the potential application of chitosan for these purposes.

This work intends to explore the antimicrobial activity of chitosan from two sources, crustaceans and insects, against non-*Saccharomyces* yeasts involved in the first steps of grape must fermentation by comparing the results regarding their tolerance of SO_2, the antimicrobial compound traditionally added to the grape must. An evaluation of the efficacy of chitosan addition, alone or in combination with low sulphite concentrations, might be useful to suggest new approaches for the production of low-sulphite wine. An evaluation of the suitability of chitosan obtained from an unconventional source, such as insects, will provide new information for the future applications of this natural compound as an antimicrobial compound for the production of wine with low sulphite content and for other food processing methods.

2. Materials and Methods

2.1. Yeast Strains

Twenty non-*Saccharomyces* yeast strains, belonging to some of the species most frequently found during grape must fermentation, were tested. The selected strains belonging to the UNIBAS Yeast Collection (UBYC), University of Basilicata (Italy), are reported in Table 1.

Table 1. Origin of the twenty non-*Saccharomyces* strains tested.

Species	Strain Code	Origin
Metschnikowia pulcherrima	AII-136 4-11; 4R1	Bees Grapes
Lachancea thermotolerans	AII-134 4-14	Bees Grapes
Pichia kluyveri	AII-110	Bees
Pichia kudriavzevii	AII-177 4-16	Bees Grapes
Pichia anomala	AII-186	Bees
Candida zemplinina	TSE FCB6	Grapes Fruit
Hanseniaspora uvarum	1P3; AP1	Grapes
Hanseniaspora guilliermondii	2R9; TM5-2	Grapes
Hanseniaspora osmophila	ND1	Grapes
Torulaspora delbrueckii	425; LC2-1	Grapes
Zygosaccharomyces bailii	CR1; CR2	Grapes

The strains were maintained on slants containing YPD medium (2% glucose, 2% peptone, 1% yeast extract; Oxoid, Hampshire, UK) with 2% agar (Oxoid, Hampshire, UK) and stored at 4 °C.

2.2. Evaluation of SO_2 and Chitosan Tolerance During Inoculated Fermentations

Twenty strains were tested for tolerance to SO_2 and commercial chitosan, based on the effect of the two antimicrobial substances on the viability of these strains during grape must fermentation. Stock solutions (10 g/L) were prepared for both the antimicrobials. As regards SO_2, potassium metabisulfite (Merck KGaA, Darmstadt, Hesse, Germany) was employed and the solution was sterilized by filtration (0.2 µm). Regarding the other antimicrobial, chitosan from shrimp shells (deacetylation degree > 75%, molecular weight 190–375 kDa), purchased from Merck KGaA (Darmstadt, Hesse, Germany), was solubilized

in 1% (*v*/*v*) of glacial acetic acid 99% (Merck KGaA, Darmstadt, Hesse, Germany). The solution was stirred overnight to obtain the complete dissolution of chitosan which was sterilized at 121 °C for 15 min.

The strains were inoculated in 100 mL of pasteurized (100 °C for 20 min) "Aglianico del Vulture" grape must (240 g/L sugar). The absence of viable cells in grape must after pasteurization was verified by plate counting on Wallerstein Laboratory Nutrient Agar medium (WL; Oxoid, Hampshire, UK).

Each strain was grown in YPD broth at 26 °C for 24 h and the inoculum level for each strain was set at 1×10^4 cells/mL.

The fermentation trials were carried out in duplicate and the following conditions were tested: (a) grape must containing 50 mg/L of SO_2, the amount frequently used during cellar fermentations [31]; (b) grape must containing 100 mg/L of commercial chitosan (Merck KGaA), the amount authorized by the OIV [15] to control spoilage microorganisms in wine; (c) grape must containing 20 mg/L of SO_2 and 100 mg/L of commercial chitosan, in order to try to reduce the amount of SO_2; (d) grape must without antimicrobial compounds (positive control); (e) grape must without yeast inoculum and antimicrobials (negative control).

The flasks were incubated at 26 °C, without agitation, for 48 h to evaluate the antimicrobial activity of each treatment during the first fermentation step. To this end, the viability of inoculated starters was checked by microbial viable counts at T_0 and T_{48}, using WL agar medium. Dilution plates containing statistically representative numbers of colonies were counted.

For each antimicrobial treatment, percentage strain resistance was calculated as the ratio between the number of generations of the treated sample and the number of generations of the positive control (without treatment). The number of generations after 48 h of incubation was calculated using the formula $N = (\log n_{t48} - \log n_{t0})/\log 2$, where n_{t48} is the number of colony-forming units (CFU)/mL after 48 h of fermentation and n_{t0} is the number of CFU/mL at the beginning of the fermentation.

2.3. Screening for Resistance to Commercial and Insect-Based Chitosan

Twenty non-*Saccharomyces* yeasts were tested to determine the resistance level of two types of chitosan: commercial from shrimp shells purchased from Merck KGaA (Darmstadt, Hesse, Germany) and insect-based chitosan (deacetylation degree > 90%, molecular weight 80–100 kDa) extracted from *Hermetia illucens* pupal exuviae. Raw samples were obtained from Xflies s.r.l (Potenza, Italy). Appropriate volumes of the stock solutions (10 g/L) of both chitosan types, solubilized in 1% *v*/*v* acetic acid, were added to an agarized medium composed of pasteurized grape must (Aglianico del Vulture) supplemented with aqueous agar solution (2% agar) to obtain a range of concentrations: 100, 200, 300, and 400 mg/L. The yeast strains were inoculated at an initial concentration of approximately 1×10^6 cells/mL and the plates were incubated for 48 h. Chitosan-free medium inoculated with the test strains was used as the control. For each strain, the resistance level was expressed as the maximum dose at which microbial growth was observed. All experiments were conducted in duplicate.

2.4. Use of Antimicrobial Treatments During Inoculated Fermentations at Laboratory Scale

This step was performed to evaluate the efficiency of the tested antimicrobial treatments in wine fermentation at a laboratory scale.

The fermentations were performed in flasks containing 2 L of fresh Aglianico del Vulture grape must and skins (255.8 g/L of sugar content; pH = 3.48; total acidity: 5.47 g/L), kindly supplied by the Cantina di Venosa cellar (Basilicata, Italy) during the 2023 vintage. The grape must was added to the following antimicrobials: 50 mg/L of SO_2; 100 mg/L of commercial chitosan; 100 mg/L of insect-based chitosan; 20 mg/L of SO_2 and 100 mg/L of commercial chitosan; 20 mg/L of SO_2 and 100 mg/L of insect-based chitosan.

After antimicrobial treatment, the flasks were inoculated with the 4LBI-3 *Saccharomyces cerevisiae* strain (inoculum level of 6×10^6 cells/mL). This was a selected indigenous strain

isolated from "Aglianico del Vulture" grape must and belonging to the UBYC [32]. Fermentation was conducted in duplicate, and the fermentation temperature was maintained at 20 °C.

Fermentation kinetics were monitored daily by measuring sugar consumption. The main wine chemical parameters (sugar content, ethanol production, total acidity, volatile acidity, pH, and malic acid) were measured daily using a Fourier Transform Infrared WineScan instrument (OenoFoss™, Hillerød, Denmark). The evolution of the yeast population was monitored at different times (1, 2, 4, 7, 10, and 17 days of fermentation) by viable plate count using two media: WL Nutrient Agar medium (Oxoid, Hampshire, UK), a differential medium useful for preliminary discrimination of the main wine yeast species, and Lysine Agar medium (Oxoid, Hampshire, UK), a medium selective for non-*Saccharomyces* yeasts. The plates were incubated at 26 °C for five days, after which the statistically representative dilution plates were counted. The colonies showing *S. cerevisiae* morphology were subjected to amplification of the interdelta region with a δ2/δ12 primer pair [33], following a previously described protocol [34], to check the dominance level of the inoculated starter.

2.5. Wine Analysis

At the end of the alcoholic fermentation, all the produced wines were analyzed for different parameters.

2.5.1. Chemical Analysis

Chemical wine parameters (sugar content, ethanol production, total and volatile acidity, pH, and malic acid) were measured using a Fourier Transform Infrared WineScan instrument (OenoFoss™, Hillerød, Denmark). Total acidity was expressed as the sum of fixed acids (tartaric, malic, citric, succinic, and lactic acids) and volatile acid (acetic acid).

2.5.2. Chromatic Characteristics

Chromatic characteristics were detected using the method described in the "Compendium of International Methods of Analysis" [35]. The wavelength (λ) at 420, 520, and 620 nm of a 1 mL wine sample was measured using the spectrophotometer Spectrostar^{nano} (BMG LABTECH, Ortenberg, Germany) to evaluate the principal chromatic characteristics of wine, which are the intensity and hue. The intensity was calculated as the sum of OD_{420}, OD_{520}, and OD_{620}, and the hue as the ratio of OD_{420} and OD_{520}.

2.5.3. Polyphenols Content Detection

The total polyphenol content was measured spectrophotometrically using the Folin–Ciocalteu (Merck KGaA, Darmstadt, Hesse, Germany) reaction with an experimental wine sample measured at 765 nm against a blank, according to the protocol described by Singleton et al. [36]. The total polyphenol compound concentration was quantified using a calibration curve ($R^2 > 0.995$) of gallic acid solutions at concentrations of 100, 200, 300, 400, and 500 mg/L (Merck KGaA, Darmstadt, Hesse, Germany). The measurements were performed in duplicate, and the results are expressed as milligrams of gallic acid equivalent (GAE) per litre of wine (mg GAE/L).

2.5.4. Antioxidant Activity

The antioxidant activity of the wines was evaluated via the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, as reported by Sánchez-Moreno et al. [37].

The DPPH assay is based on the inhibition of the radical DPPH by the antioxidant component of wine and was performed as follows: a 0.6 mM DPPH solution in ethanol (99%, Merck KGaA, Darmstadt, Hesse, Germany) was prepared and stirred for at least two hours, in order to wait for radicalization, and diluted with ethanol to obtain an absorbance level of 1 at 515 nm. A total of 10 µL of each wine was mixed with 990 µL of diluted DPPH radical solution, and after reaction for 15 min, the absorbance was measured at 515 nm. The

reducing capacity was calculated with reference to the Trolox calibration curve ($R^2 > 0.997$) and the results were expressed as a percentage of DPPH reduction.

2.5.5. Aromatic Compounds

The main secondary compounds affecting wine aroma, such as acetaldehyde, ethyl acetate, acetoin, and higher alcohols, were analyzed using an Agilent 7890 A gas chromatograph equipped with a flame ionization detector (FID) connected to Agilent Chemstation software for data analysis, as described by Capece et al. [38]. One microlitre of sample was injected into a glass column packed with 80/120 Carbopack BAW 5% and Carbowax 20 M (Supelco, Bellefonte, PA, USA).

The oven was run from 70 °C to 130 °C at a ramp rate of 5 °C/min, and then up to 180 °C at a ramp rate of 7 °C/min. Helium was used as the carrier gas at a flow rate of 20 mL/min. The levels of secondary compounds were determined by internal standardization.

2.6. Statistical Analysis

Statistical analysis of the entire dataset was performed using Paleontological Statistics (PAST) software [39]. One-Way Analysis of Variance (ANOVA) followed by a *post hoc* comparison (Tukey's HSD test) was carried out; p values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Influence of SO_2 and Chitosan on Viability of Non-Saccharomyces Strains During Inoculated Fermentations

The effects of chitosan and SO_2 (tested both alone and in combination) against the twenty non-*Saccharomyces* strains were evaluated during inoculated fermentation by determining the influence of these antimicrobials on the viability of yeast strains in the first step of fermentation inoculated with each strain.

The effect of antimicrobial treatments is reported in Table 2, where the resistance percentage is based on the ratio between the generation number of treated and control samples in the first 48 h of fermentation. Generally, the tested strains were more resistant to chitosan than SO_2, except for the *M. pulcherrima* (particularly 4-11 and 4R1) and *T. delbrueckii* strains. For some strains, no statistically significant differences among the three treatments were found, such as AII-136 (*M. pulcherrima*), TSE (*C. zemplinina*) and AII-186 (*P. anomala*). With regard to the use of the combined treatment involving chitosan (100 mg/L) and a reduced amount of SO_2 (20 mg/L), the resistance percentage was similar to that observed for the single treatment, mainly for chitosan addition. Statistically significant differences were only found in a few strains among the three treatments; these strains, such as 4-11, AP1, 2R9, AII-134, 4-14, FCB6, exhibited resistance percentages with intermediate values between the two single treatments.

Our results demonstrated that the effectiveness of the tested antimicrobials appears to be related not only to the species, but also to the strain of the analyzed yeasts. One *M. pulcherrima* strain (AII-136) was highly sensitive to all antimicrobials, whereas two strains (4-11 and 4R1) were less tolerant to chitosan and highly resistant to SO_2. The effect of chitosan treatment on *M. pulcherrima* has also been reported by other authors [29]. Barbosa et al. [40] found that the majority of *M. pulcherrima* isolates analyzed in their study tolerated the highest chitosan concentration tested (1 g/L), but resistance was evaluated in YPD agar medium (with pH adjusted to 3.5), while it was reported that the efficacy of chitosan decreased under winemaking conditions. The high SO_2 resistance of *M. pulcherrima* has been reported in other studies, using different strains and methodologies [40–42].

Table 2. Resistance percentage of twenty non-*Saccharomyces* strains to antimicrobial treatment, based on the ratio between the generation number of treated and control samples in the first 48 h of fermentation.

Species	Strain Code	50 mg/L SO_2	100 mg/L Chitosan	20 mg/L SO_2 + 100 mg/L Chitosan
M. pulcherrima	AII-136	17.91 ± 0.88	18.68 ± 1.02	16.22 ± 1.24
	4-11	74.92 ± 3.40 ^{a}	19.41 ± 0.08 ^{b}	44.76 ± 0.96 ^{c}
	4R1	75.45 ± 3.31 ^{a}	37.13 ± 3.74 ^{b}	29.29 ± 1.02 ^{b}
Z. bailii	CR-1	92.40 ± 3.22 ^{a}	83.16 ± 1.55 ^{b}	80.83 ± 0.98 ^{b}
	CR-2	98.71 ± 1.83 ^{a}	90.32 ± 2.38 ^{b}	89.51 ± 1.54 ^{b}
T. delbrueckii	425	72.54 ± 2.25 ^{a}	48.16 ± 1.44 ^{b}	48.49 ± 2.45 ^{b}
	LC2-1	93.81 ± 1.37 ^{a}	74.49 ± 1.61 ^{b}	96.49 ± 2.99 ^{a}
H. uvarum	AP1	0.10 ± 0.04 ^{a}	81.12 ± 0.71 ^{b}	16.71 ± 2.14 ^{c}
	1P3	76.91 ± 3.08 ^{a}	99.95 ± 0.11 ^{b}	99.45 ± 0.78 ^{b}
H. guilliermondii	2R9	23.30 ± 0.42 ^{a}	99.93 ± 0.11 ^{b}	86.20 ± 0.88 ^{c}
	TM5-2	6.36 ± 0.65 ^{a}	59.15 ± 2.38 ^{b}	60.41 ± 2.36 ^{b}
H. osmophila	ND1	56.34 ± 2.43 ^{a}	81.99 ± 3.71 ^{b}	73.32 ± 4.69 ^{b}
L. thermotolerans	AII-134	22.31 ± 2.24 ^{a}	82.63 ± 3.35 ^{b}	59.43 ± 1.46 ^{c}
	4-14	41.94 ± 1.82 ^{a}	80.15 ± 2.03 ^{b}	58.39 ± 2.97 ^{c}
C. zemplinina	TSE	90.50 ± 5.31	99.88 ± 0.17	91.67 ± 1.89
	FCB6	52.20 ± 3.05 ^{a}	99.40 ± 0.71 ^{b}	78.88 ± 3.32 ^{c}
P. kudriavzevii	AII-177	74.49 ± 1.91 ^{a}	87.42 ± 2.75 ^{b}	75.48 ± 1.28 ^{a}
	4-16	61.62 ± 3.65 ^{a}	85.02 ± 3.14 ^{b}	79.09 ± 1.99 ^{b}
P. anomala	AII-186	76.02 ± 3.11	83.23 ± 0.29	84.01 ± 1.28

Data are the means of duplicate experiments ± standard deviation. Superscript letters correspond to significant differences ($p < 0.05$) among different treatments for each strain.

All the *Hanseniaspora* strains tested in our study were unaffected by chitosan addition, in agreement with previous findings showing that chitosan treatment was not able to prevent the development of *H. uvarum* [29,43]. In particular, the 1P3 *H. uvarum* strain exhibited very high tolerance to both chitosan and SO_2. Other studies have reported high variability in SO_2 tolerance among *Hanseniaspora* yeasts [44,45], and strains characterized by tolerance at concentrations much higher than those typically used in winemaking have been described [46].

These findings contradict the general concept that non-*Saccharomyces* yeasts are sensitive to SO_2 doses commonly used in vinification, a major question in the main roles of SO_2 use in winemaking.

3.2. Evaluation of Strain Resistance to Commercial and Insect-Based Chitosan

Figure 1 illustrates the resistance levels, corresponding to the highest dose tolerated (mg/L) by twenty non-*Saccharomyces* yeast strains when treated with commercial and insect-based chitosan. Notably, very few studies have evaluated the level of resistance to chitosan among yeast strains belonging to different yeast species frequently encountered during spontaneous wine fermentation, such as *C. zemplinina*, *T. delbrueckii*, and *L. thermotolerans* [29,47]. Furthermore, the antimicrobial activity of an innovative and sustainable source of chitosan, which was insect-based chitosan, was first evaluated in this study.

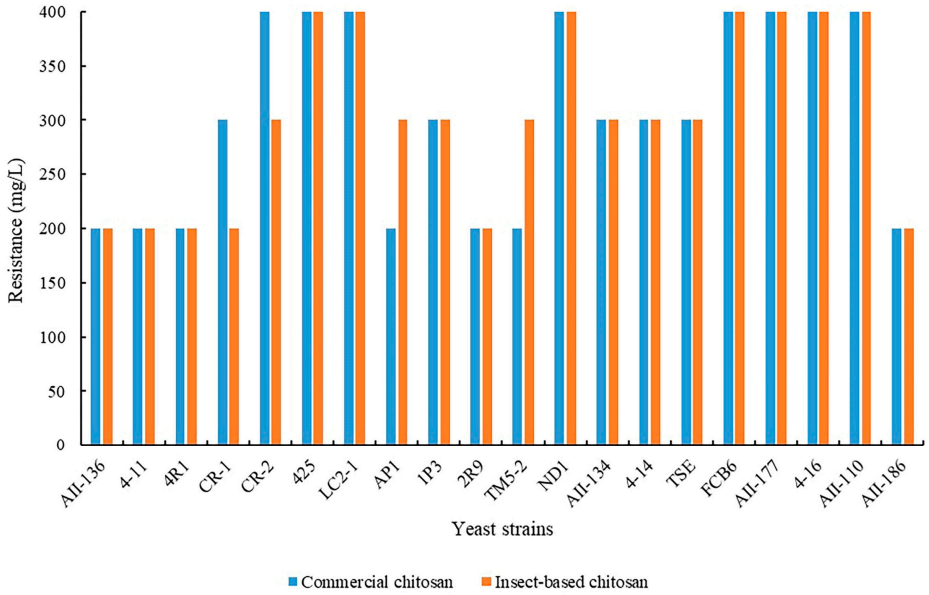

Figure 1. Non-*Saccharomyces* resistance level to commercial and insect-based chitosan, measured on agarized grape must.

As shown in Figure 1, most yeast strains exhibited similar behaviour to both types of chitosan. Some of them, such as 425 and LC2-1 (*T. delbrueckii*), ND1 (*H. osmophila*), FCB6 (*C. zemplinina*), 4-16 (*P. kudriavzevii*), and AII-110 (*P. kluyveri*), showed the highest resistance level, tolerating up to 400 mg/L for both types of chitosan. This indicated strong resistance capabilities, irrespective of the type of chitosan used. The lowest resistance levels were observed in strains AII-136, 4-11, and 4R1 (*M. pulcherrima*), 2R9 (*H. guilliermondii*), and AII-186 (*P. anomala*), where microbial growth stopped as early as 200 mg/L of the two compounds. Some strains, such as CR-1 and CR-2 (*Z. bailii*), displayed differences in resistance levels to the two antimicrobials, showing a higher resistance to the commercial chitosan compared to the insect-based one, with the latter having better efficacy. The opposite behaviour, however, was observed in strains AP1 (*H. uvarum*) and TM5-2 (*H. guilliermondii*), where commercial chitosan had better antimicrobial activity than insect-based chitosan.

These data suggest that insect-based chitosan generally performs on par with commercial chitosan across a wide range of yeast strains, whereas only a few strains exhibit different resistance levels depending on the chitosan type used. The antimicrobial activity of chitosan is correlated with some chemical characteristics, such as deacetylation degree and molecular weight, with stronger activity for higher deacetylation degrees and low molecular weights, and under acidic conditions for its amino groups to be charged [48,49]. Low-molecular-weight (LWM) chitosan is better able to penetrate bacterial cell walls, compromising membrane integrity and disrupting cell metabolism [50], while the degree of deacetylation (DD) influences the positive charge of chitosan. A high DD increases the positive charge density, enhancing electrostatic interactions with the negatively charged bacterial cell membranes. This interaction damages the cell wall, causing the loss of ions and components vital to the cell [51].

By comparing the chemical characteristics of the two chitosan types, we expected a higher antimicrobial activity of insect-based chitosan (deacetylation degree > 90%, molecular weight 80–100 kDa) [14] than commercial chitosan (deacetylation degree > 75%, molecular weight 190–375 kDa). However, other factors, including the type of microorganism, can affect its antimicrobial activity, and to achieve the highest antimicrobial activity, the optimum conditions of chitosan application should be investigated and tested before its application [52].

Regarding behaviour among strains of some species, all *M. pulcherrima* strains showed the same resistance level, which was 200 mg/L, independent of the chitosan source. The low tolerance of *M. pulcherrima* to chitosan treatment of apple juice has already been reported [53], but no further data on the chitosan tolerance of this species are available in the literature. Strains of other species such as *Z. bailii* (CR-1 and CR-2) and *C. zemplinina* (TSE and FCB6) showed strain-dependent resistance. The resistance level of the CR-1 strain (300 and 200 mg/L for commercial and insect-based chitosan, respectively) was lower than that of the CR-2 strain (400 and 300 mg/L for commercial and insect-based chitosan, respectively). Our findings are in accordance with those of other studies [30,54], which reported that *Z. bailii* is susceptible to chitosan at 0.1 and 0.4 g/L. As previously reported [47], yeasts with high fermentative attitudes, such as *T. delbrueckii*, showed remarkable resistance to chitosan. It has been found that chitosan may be an effective fungicidal agent, but different studies have reported that rather high concentrations are required to inhibit yeast growth [55–58].

Overall, these results indicated different behaviour among different yeast strains/species, which might be related to the different composition of the cell surface, which is one of the main action sites of chitosan. Indeed, it is well known that cell surface properties, cell wall composition, such as polysaccharide content, and molecular organization of the cell wall vary among different yeast species and strains [59,60].

3.3. Effect of Antimicrobial Treatments During Inoculated Lab-Scale Fermentations

This step was performed to evaluate the efficiency of different antimicrobial treatments during laboratory-scale fermentation, inoculated with the selected *S. cerevisiae* strain 4LBI-3. The antimicrobial treatments tested were commercial chitosan, SO_2, and insect-based chitosan. The three antimicrobials were used alone and in combination with low SO_2 levels (20 mg/L).

Fermentation kinetics were monitored by measuring the sugar consumption and ethanol production (Figure 2). In general, all fermentations were completed and lasted seventeen days. The final sugar level was less than 1 g/L and ethanol was approximately 15% *v*/*v* for all wines. Fermentation with 50 mg/L SO_2 showed a different trend than the others during the first days of the process, with slower sugar consumption and ethanol production. Opposite results were reported by other authors, who found slower fermentation rates in grapes treated with chitosan [43,61].

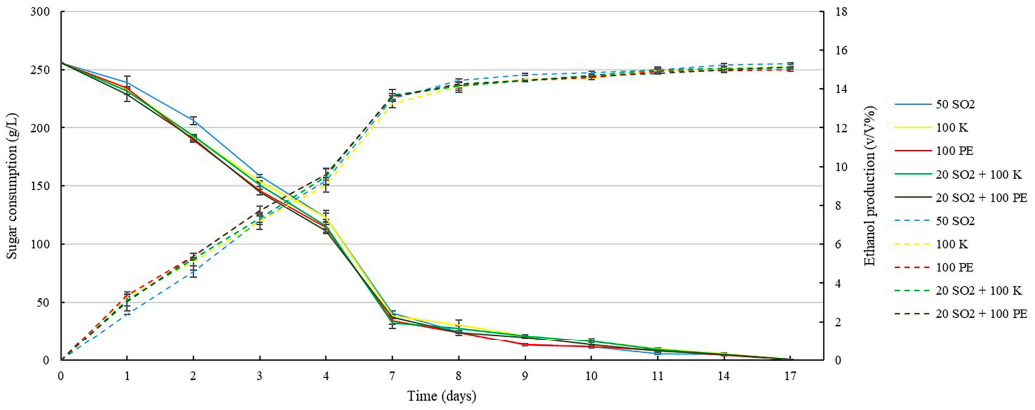

Figure 2. Sugar consumption (solid line) and ethanol production (dashed lines) during inoculated fermentations at laboratory scale containing different antimicrobials: 50 mg/L of SO_2 (50 SO_2); 100 mg/L of commercial chitosan (100 K); 100 mg/L of insect-based chitosan (100 PE); 20 mg/L of SO_2 and 100 mg/L of commercial chitosan (20 SO_2 + 100 K); 20 mg/L of SO_2 and 100 mg/L of insect-based chitosan (20 SO_2 + 100 PE).

However, by the fourth day of fermentation, the differences were reduced, and the fermentation trend was similar under all conditions. In all cases, the alcoholic fermentations

were completed, regardless of the addition of chitosan and/or SO_2. However, as previously reported [30], chitosan did not alter the fermentative performance of *S. cerevisiae* starters, although some authors [47] reported lengthening of the lag phase due to the initial killing of part of the yeast population, demonstrating its potential applicability in wine fermentation.

To investigate the antimicrobial effect of all treatments, yeast population dynamics during fermentation were evaluated by viable plate count after 1, 2, 4, 7, 10, and 17 days of fermentation. In general, the trend was similar for all fermentations, showing a decrease in non-*Saccharomyces* cell viability over time; however, after 7 days of fermentation, no colonies of non-*Saccharomyces* yeasts were found for any of the conditions. The viable count of non-*Saccharomyces* yeasts, shown in Figure 3A, was affected by the treatment. On day 1, 24 h after the starter inoculum and antimicrobial addition, the lowest level of viable non-*Saccharomyces* cells was found in the fermentation with only sulphur dioxide, while the highest level was detected in the fermentation with insect-based chitosan, both alone and in combination with SO_2. The lowest concentration of indigenous microflora observed in SO_2-added fermentation might explain the lower initial rate of fermentation observed in this sample compared to other antimicrobial treatments (Figure 2).

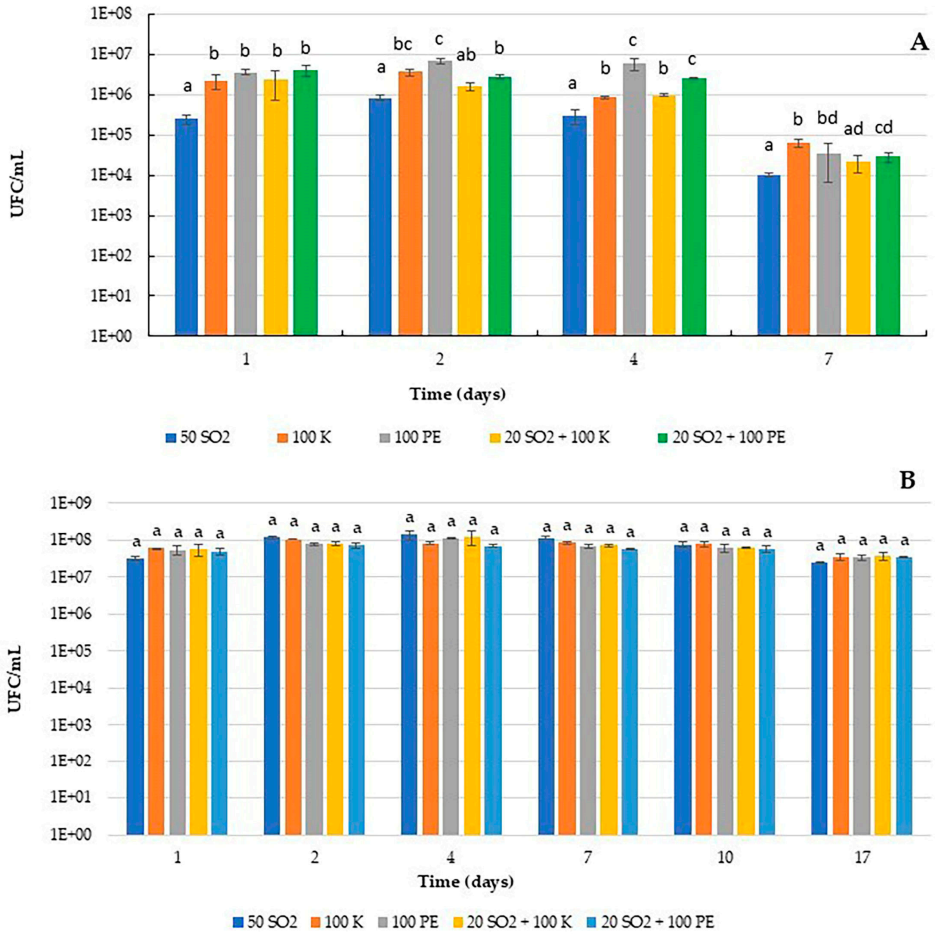

Figure 3. Viable cell population of non-*Saccharomyces* (**A**) and *S. cerevisiae* (**B**) yeasts detected at different times of fermentation inoculated with *S. cerevisiae* 4LBI-3 and containing different antimicrobials: 50 mg/L of SO_2 (50 SO_2); 100 mg/L of commercial chitosan (100 K); 100 mg/L of insect-based chitosan (100 PE); 20 mg/L of SO_2 and 100 mg/L of commercial chitosan (20 SO_2 + 100 K); 20 mg/L of SO_2 and 100 mg/L of insect-based chitosan (20 SO_2 + 100 PE). Data are the means of duplicate experiments ± standard deviation. Letters on plot bars indicate significant differences ($p < 0.05$) among various treatments.

On day 2, after 48 h, the fermentation with the use of SO_2 alone again showed the lowest level of viable non-*Saccharomyces* cells, but this value was slightly higher than that on day 1, probably due to the adaptation of the yeasts to the medium. Furthermore, both types of chitosan showed lower activity than SO_2, but the combined use with sulphur dioxide showed a better effect than the use of chitosan alone for both. Previous findings [30] reported the highest efficacy of SO_2 addition (40 mg/L) for the inhibition of non-*Saccharomyces* yeasts in comparison to chitosan treatments, but a reduction in the viable cell number below the detection limit was observed on the fourth day of fermentation, whereas in our study, it occurred on the seventh fermentation day. Other authors [43] reported a lower inhibitory effect of chitosan with respect to SO_2 on non-*Saccharomyces* yeasts, although the dose used by these authors (400 mg/L) was higher than ours.

On day 4, when the sugar content was reduced by approximately 50% (Figure 2), a reduction in viable cells was observed only for SO_2 and commercial chitosan treatments, whereas for the other fermentations, the number of viable cells was similar to that observed on the second day of fermentation.

Finally, on day 7, the highest reduction in viable cells was observed for all the trials, and this result could also be related to the ethanol content reached at this point, which was approximately 9% (*v*/*v*) for almost all fermentations (Figure 2). In this step, the highest reduction was observed in the case of SO_2 treatment, in which the non-*Saccharomyces* population reached 1×10^4 CFU/mL, whereas the highest viable count was found in the case of treatment with commercial chitosan (6.5×10^4 CFU/mL). Similar numbers of viable cells were found among the other treatments (ranging between 2.1×10^4 and 3.5×10^4 CFU/mL).

Similar results [30] were reported by other authors, who found a drastic decline in the non-*Saccharomyces* population in fermentations treated with 0.2, 0.4, and 0.6 g/L chitosan on the eighth day of fermentation and the complete inactivation of the non-*Saccharomyces* yeasts after six days when chitosan was applied in combination with SO_2.

Regarding the evolution of the *S. cerevisiae* population (Figure 3B), the trend was similar in all fermentations, and the number of viable cells did not show differences among the treatments with different antimicrobial compounds. This may indicate that neither sulphur dioxide nor commercial or insect-based chitosan interferes with the normal development of *S. cerevisiae*. Our results agree with those of other studies that reported that the addition of chitosan did not significantly alter the viability and fermentative performance of *S. cerevisiae* [30,62].

The number of viable cells at the end of alcoholic fermentation ranged between 2.4×10^7 and 3.75×10^7 CFU/mL. The analysis of interdelta profiles of colonies isolated at the different sampled points revealed that all the isolates showed the same molecular profile of the starter, indicating a complete dominance of the 4LBI-3 strain during the overall process.

3.4. Analyses of Experimental Wines Obtained with Different Antimicrobial Treatments

The general oenological parameters and main volatile compounds detected in the experimental wines subjected to different antimicrobial treatments are shown in Table 3. Regarding alcohol content, similar values (ranging between 14.98 and 15.31% *v*/*v*) were found in all wines, with the lowest level detected in the wine obtained by grape must containing insect-based chitosan. The highest ethanol level was found in wine produced with SO_2 addition, which had the lowest residual sugar content. No differences were found among the wines in total acidity, whereas the volatile acidity varied among the different treatments, although the values were within the acceptable level (less than 1 g/L). The highest level was detected in wine produced by adding insect-based chitosan (alone and in combination with SO_2). This result might be correlated with the highest non-*Saccharomyces* counts detected during the first seven fermentation days for this sample (Figure 3A). Indeed, it is well known that non-*Saccharomyces* yeast species prevalent in the

first steps of fermentation (such as *H. uvarum*/*Kloeckera apiculata*) are high producers of volatile acidity [41].

Table 3. Main secondary compounds and main chemical parameters of experimental wine obtained by Aglianico del Vulture grape must fermentation inoculated with *S. cerevisiae* (4LBI-3) with the use of different antimicrobial treatments.

	50 mg/L SO_2	100 mg/L Commercial Chitosan	100 mg/L Insect-Based Chitosan	20 mg/L SO_2 + 100 mg/L Commercial Chitosan	20 mg/L SO_2 + 100 mg/L Insect-Based Chitosan
Ethanol	15.31 ± 0.04 ^{a}	15.18 ± 0.10 ^{ab}	14.98 ± 0.05 ^{b}	15.10 ± 0.08 ^{ab}	15.12 ± 0.04 ^{ab}
Glucose + fructose	0.30 ± 0.06 ^{a}	0.56 ± 0.04 ^{b}	0.45 ± 0.01 ^{ab}	0.40 ± 0.07 ^{ab}	0.50 ± 0.04 ^{b}
Total acidity	9.35 ± 0.14	8.85 ± 0.10	9.03 ± 0.11	9.02 ± 0.13	8.85 ± 0.21
Volatile acidity	0.41 ± 0.02 ^{a}	0.44 ± 0.02 ^{a}	0.65 ± 0.01 ^{b}	0.44 ± 0.06 ^{ac}	0.59 ± 0.05 ^{bc}
Malic acid	1.37 ± 0.01 ^{a}	1.35 ± 0.01 ^{a}	1.23 ± 0.06 ^{b}	1.30 ± 0.01 ^{ab}	1.21 ± 0.00 ^{b}
Acetaldehyde	28.23 ± 0.40	30.48 ± 3.92	29.24 ± 1.30	26.40 ± 1.11	26.92 ± 0.34
Ethyl acetate	29.78 ± 0.51 ^{a}	47.08 ± 2.07 ^{bc}	49.94 ± 4.28 ^{b}	38.51 ± 3.30 ^{ac}	36.90 ± 1.38 ^{ac}
n-Propanol	14.28 ± 0.04 ^{a}	32.35 ± 2.09 ^{b}	28.31 ± 1.85 ^{bc}	24.33 ± 0.12 ^{cd}	22.09 ± 0.37 ^{d}
Isobutanol	27.74 ± 0.38 ^{a}	26.23 ± 1.89 ^{ab}	31.53 ± 0.33 ^{ac}	27.00 ± 0.31 ^{a}	30.95 ± 1.23 ^{ac}
n-butanol	12.44 ± 0.16	11.95 ± 1.68	11.58 ± 0.39	11.95 ± 0.21	12.19 ± 0.48
Acetoin	6.02 ± 0.55	5.80 ± 0.63	4.70 ± 0.01	4.84 ± 0.28	4.83 ± 0.12
2-methyl-1-butanol	84.04 ± 4.01 ^{a}	85.14 ± 0.74 ^{a}	71.91 ± 2.93 ^{b}	81.02 ± 0.32 ^{ab}	71.35 ± 2.63 ^{b}
3-methyl-1-butanol	221.71 ± 2.08 ^{a}	207.26 ± 4.00 ^{ab}	196.66 ± 6.69 ^{b}	209.05 ± 1.62 ^{ab}	195.30 ± 1.91 ^{b}
Total polyphenols	1862.27 ± 48.82 ^{a}	1446.58 ± 25.05 ^{b}	1659.48 ± 1.28 ^{c}	1703.28 ± 37.97 ^{cd}	1790.10 ± 6.90 ^{ad}
% DPPH reduction	40.48 ± 0.81 ^{a}	33.66 ± 0.67 ^{b}	36.53 ± 0.67 ^{bc}	38.74 ± 1.60 ^{ac}	40.04 ± 0.02 ^{ac}

Data are the means of duplicate experiments ± standard deviation. Superscript letters correspond to significant differences ($p < 0.05$) among different treatments for each parameter. The main oenological parameters (glucose + fructose, total and volatile acidities, malic acid) are expressed as g/L, ethanol as % *v*/*v*, volatile compounds as mg/L, and total polyphenols as mg GAE/L.

Regarding the content of the main volatile compounds affecting wine aroma, despite different treatments, the wines showed no significant differences in the levels of acetaldehyde, n-butanol, or acetoin. Statistically significant differences were found for the other compounds analyzed. In particular, the wine treated with only SO_2 showed the lowest level of ethyl acetate and n-propanol compared to all the others. With regard to the content of 3-methyl-1-butanol, the lowest level was found in wine treated with insect-based chitosan (alone and in combination with SO_2), with statistically significant differences compared to wine treated with 50 mg/L of sulphur dioxide. Similar behaviour was observed for 2-methyl-1-butanol. Other authors [61] found higher levels of 3-methyl-1-butanol in SO_2-added wines than in chitosan-treated samples, confirming previous findings that SO_2 presence during fermentation favours a rapid consumption of amino acids [63,64].

The total polyphenols content and antioxidant activity of the experimental wines were also analyzed to evaluate the influence of the antimicrobials used on these parameters (Table 3). Regarding the polyphenol content measured by the Folin–Ciocalteu reaction, variability was observed among the experimental wines. The sample treated with 50 mg/L of SO_2 showed the highest polyphenol content around 1862.3 mg GAE/L, whereas the sample treated with the two types of chitosan alone (both commercial and insect-based) showed the lowest level, ranging from 1446.5 to 1659.5 mg GAE/L. The wine produced with the combined use of sulphur dioxide and commercial/insect-based chitosan exhibited a medium level of polyphenol content, which was not very different from the other wines, ranging from 1703 to 1790 mg GAE/L.

A similar trend was observed for the evaluation of antioxidant activity, which was calculated as the percentage of DPPH reduction. The highest level was observed in the experimental wine produced with SO_2 alone, and as found for the polyphenol content, the lowest value was obtained in samples treated with both chitosan types alone.

In addition, a statistically significant ($p < 0.05$) correlation (r = 0.977) between polyphenol content and % DPPH reduction was observed.

This result may be correlated with the high affinity of chitosan for phenolic compounds in wine, as reported by other authors [65]. Indeed, in wine, owing to its low pH, chitosan is a polymer with a high positive charge density, which allows the formation of an efficient complex (by means of non-covalent forces, such as hydrophobic or electrostatic interactions and/or hydrogen bonding) between chitosan and polyphenols and the formation of soluble complexes that can aggregate, with subsequent precipitation [66,67].

The chromatic characteristics of the experimental wines are shown in Figure 4, where the colour intensity was calculated as the sum of OD_{420}, OD_{520}, and OD_{620} and the hue as the ratio between OD_{420} and OD_{520}. Statistically significant differences in colour intensity were found among the different treatments. Wines treated with chitosan showed higher intensity than those treated with SO_2. The lowest colour intensity was observed for the wine treated with the combined use of SO_2 and commercial chitosan, whereas the use of insect-based chitosan + SO_2 resulted in wine with a colour intensity similar to that of wine obtained from insect-based chitosan alone, while some studies reported that chitosan appreciably reduces colour intensity, but the reduction is dependent on the doses of chitosan applied [68].

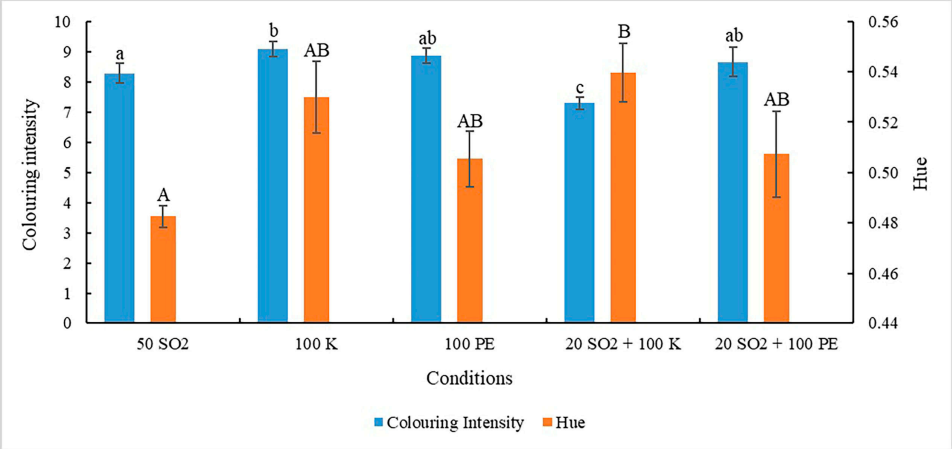

Figure 4. Colour intensity and hue of wine produced with the use of different antimicrobial treatments: 50 mg/L of SO_2 (50 SO_2); 100 mg/L of commercial chitosan (100 K); 100 mg/L of insect-based chitosan (100 PE); 20 mg/L of SO_2 and 100 mg/L of commercial chitosan (20 SO_2 + 100 K); 20 mg/L of SO_2 and 100 mg/L of insect-based chitosan (20 SO_2 + 100 PE). Data are the means of duplicate experiments ± standard deviation. Letters on plot bars indicate significant differences ($p < 0.05$) among various treatments. Lowercase letters indicate differences in colour intensity and capital letters indicate differences in hue.

With regard to hue, the lowest value was found in the wine obtained with the use of 50 mg/L of SO_2, while the wine from the combined use of SO_2 and commercial chitosan showed the highest value. No statistically significant differences were observed among the other wines.

4. Conclusions

In this study, the effects of two chitosan types, commercial and insect-based, on non-*Saccharomyces* yeasts associated with grape must fermentation were evaluated. The overall results demonstrated that the wine yeasts tested in this study showed medium/high resistance to chitosan, but a variable behaviour in the function of the strain or species was observed. Insect-based chitosan generally performs on par with commercial chitosan across a wide range of yeast strains, whereas only a few strains exhibit different resistance levels depending on the type of chitosan used.

The addition of the two chitosan types during grape must fermentation inoculated with *S. cerevisiae* showed lower antimicrobial activity than that of SO_2, but their combined use with sulphur dioxide showed a better effect than the use of chitosan alone for both. However, chitosan treatment did not seem to inhibit *S.cerevisiae*, confirming the potential use of this compound during wine fermentation.

Further investigations are currently being carried out to better understand the antimicrobial mechanism and identify the elements involved in microorganism sensitivity and/or tolerance to insect-based chitosan. The evaluation of the suitability of chitosan obtained from a sustainable source, such as insects, will provide new information for future applications of this natural compound to produce wine with low sulphite content and for other food processing.

Author Contributions: F.T.: conceptualization, data curation, investigation, methodology, writing—original draft. R.P.: data curation, formal analysis, investigation, methodology. G.S.: data curation, methodology, writing—review and editing. C.S.: writing—review and editing. R.S.: writing—review and editing. P.F.: writing—review and editing. A.C.: conceptualization, project administration, resources, supervision, writing—original draft, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Mas, A.; Beltran, G.; Torija, M.J. Microbiological Control of Alcoholic Fermentation. *Ecocycles* **2020**, *6*, 57–72. [CrossRef]
2. Guerrero, R.F.; Cantos-Villar, E. Demonstrating the Efficiency of Sulphur Dioxide Replacements in Wine: A Parameter Review. *Trends Food Sci. Technol.* **2015**, *42*, 27–43. [CrossRef]
3. Ailer, Š.; Jakabová, S.; Benešová, L.; Ivanova-Petropulos, V. Wine Faults: State of Knowledge in Reductive Aromas, Oxidation and Atypical Aging, Prevention, and Correction Methods. *Molecules* **2022**, *27*, 3535. [CrossRef] [PubMed]
4. Vally, H.; Misso, N.L.A.; Madan, V. Clinical Effects of Sulphite Additives. *Clin. Exp. Allergy* **2009**, *39*, 1643–1651. [CrossRef]
5. Vally, H.; Misso, N.L.A. Adverse Reactions to the Sulphite Additives. *Gastroenterol. Hepatol. Bed Bench* **2012**, *5*, 16–23.
6. Garcia-Fuentes, A.; Wirtz, S.; Vos, E.; Verhagen, H. Short Review of Sulphites as Food Additives. *Eur. J. Nutr. Food Saf.* **2015**, *5*, 113–120. [CrossRef]
7. World Health Organization; Food and Agriculture Organization of the United Nations. *Safety Evaluation of Certain Food Additives and Contaminants: Prepared by the Seventy-Third Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Geneva, Switzerland, 8–17 June 2010*; World Health Organization: Geneva, Switzerland; Food and Agriculture Organization of the United Nations: Rome, Italy, 2011.
8. Lisanti, M.T.; Blaiotta, G.; Nioi, C.; Moio, L. Alternative Methods to SO_2 for Microbiological Stabilization of Wine. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 455–479. [CrossRef]
9. Tedesco, F.; Siesto, G.; Pietrafesa, R.; Romano, P.; Salvia, R.; Scieuzo, C.; Falabella, P.; Capece, A. Chemical Methods for Microbiological Control of Winemaking: An Overview of Current and Future Applications. *Beverages* **2022**, *8*, 58. [CrossRef]
10. Castro Marín, A.; Colangelo, D.; Lambri, M.; Riponi, C.; Chinnici, F. Relevance and Perspectives of the Use of Chitosan in Winemaking: A Review. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 3450–3464. [CrossRef]
11. Lárez Velásquez, C. Chitosan and Its Applications in Oenology. *OENO One* **2023**, *57*, 121–132. [CrossRef]
12. Pellis, A.; Guebitz, G.M.; Nyanhongo, G.S. Chitosan: Sources, Processing and Modification Techniques. *Gels* **2022**, *8*, 393. [CrossRef] [PubMed]
13. Harkin, C.; Mehlmer, N.; Woortman, D.V.; Brück, T.B.; Brück, W.M. Nutritional and Additive Uses of Chitin and Chitosan in the Food Industry. In *Sustainable Agriculture Reviews 36: Chitin and Chitosan: Applications in Food, Agriculture, Pharmacy, Medicine and Wastewater Treatment*; Springer: Berlin/Heidelberg, Germany, 2019; pp. 1–43.

14. Triunfo, M.; Tafi, E.; Guarnieri, A.; Salvia, R.; Scieuzo, C.; Hahn, T.; Zibek, S.; Gagliardini, A.; Panariello, L.; Coltelli, M.B.; et al. Characterization of Chitin and Chitosan Derived from *Hermetia illucens*, a Further Step in a Circular Economy Process. *Sci. Rep.* **2022**, *12*, 6613. [CrossRef] [PubMed]
15. *OIV-ENO-338a-2009*; International Code of Oenological Practices. Wines-Treatment Using Chitosan. International Organization of Vine and Wine (OIV): Paris, France, 2009.
16. Chinnici, F.; Natali, N.; Riponi, C. Efficacy of Chitosan in Inhibiting the Oxidation of (+)-Catechin in White Wine Model Solutions. *J. Agric. Food Chem.* **2014**, *62*, 9868–9875. [CrossRef] [PubMed]
17. Lopes, C.; Antelo, L.T.; Franco-Uría, A.; Alonso, A.A.; Pérez-Martín, R. Chitin Production from Crustacean Biomass: Sustainability Assessment of Chemical and Enzymatic Processes. *J. Clean. Prod.* **2018**, *172*, 4140–4151. [CrossRef]
18. Amaral, L.; Silva, D.; Couto, M.; Nunes, C.; Rocha, S.M.; Coimbra, M.A.; Coimbra, A.; Moreira, A. Safety of Chitosan Processed Wine in Shrimp Allergic Patients. *Ann. Allergy Asthma Immunol.* **2016**, *116*, 462–463. [CrossRef]
19. de Gier, S.; Verhoeckx, K. Insect (Food) Allergy and Allergens. *Mol. Immunol.* **2018**, *100*, 82–106. [CrossRef]
20. van Huis, A. Potential of Insects as Food and Feed in Assuring Food Security. *Annu. Rev. Entomol.* **2013**, *58*, 563–583. [CrossRef]
21. Scieuzo, C.; Franco, A.; Salvia, R.; Triunfo, M.; Addeo, N.F.; Vozzo, S.; Piccolo, G.; Bovera, F.; Ritieni, A.; Francia, A.D.; et al. Enhancement of Fruit Byproducts through Bioconversion by *Hermetia illucens* (Diptera: *Stratiomyidae*). *Insect Sci.* **2023**, *30*, 991–1010. [CrossRef]
22. Guarnieri, A.; Triunfo, M.; Scieuzo, C.; Ianniciello, D.; Tafi, E.; Hahn, T.; Zibek, S.; Salvia, R.; De Bonis, A.; Falabella, P. Antimicrobial Properties of Chitosan from Different Developmental Stages of the Bioconverter Insect *Hermetia illucens*. *Sci. Rep.* **2022**, *12*, 8084. [CrossRef]
23. Taillandier, P.; Joannis-Cassan, C.; Jentzer, J.-B.; Gautier, S.; Sieczkowski, N.; Granes, D.; Brandam, C. Effect of a Fungal Chitosan Preparation on *Brettanomyces bruxellensis*, a Wine Contaminant. *J. Appl. Microbiol.* **2015**, *118*, 123–131. [CrossRef]
24. Portugal, C.; Sáenz, Y.; Rojo-Bezares, B.; Zarazaga, M.; Torres, C.; Cacho, J.; Ruiz-Larrea, F. *Brettanomyces* Susceptibility to Antimicrobial Agents Used in Winemaking: In Vitro and Practical Approaches. *Eur. Food Res. Technol.* **2014**, *238*, 641–652. [CrossRef]
25. Petrova, B.; Cartwright, Z.M.; Edwards, C.G. Effectiveness of Chitosan Preparations against *Brettanomyces bruxellensis* Grown in Culture Media and Red Wines. *OENO ONE* **2016**, *50*, 49. [CrossRef]
26. Nardi, T.; Vagnoli, P.; Minacci, A.; Gautier, S.; Sieczkowski, N. Evaluating the Impact of a Fungal-Origin Chitosan Preparation on *Brettanomyces bruxellensis* in the Context of Wine Aging. *Wine Stud.* **2014**, *1*, 4574. [CrossRef]
27. Paulin, M.; Miot-Sertier, C.; Dutilh, L.; Brasselet, C.; Delattre, C.; Pierre, G.; Dubessay, P.; Michaud, P.; Doco, T.; Ballestra, P.; et al. *Brettanomyces bruxellensis* Displays Variable Susceptibility to Chitosan Treatment in Wine. *Front. Microbiol.* **2020**, *11*, 571067. [CrossRef] [PubMed]
28. Ferreira, D.; Moreira, D.; Costa, E.M.; Silva, S.; Pintado, M.M.; Couto, J.A. The Antimicrobial Action of Chitosan Against the Wine Spoilage Yeast *Brettanomyces/Dekkera*. *J. Chitin Chitosan Sci.* **2013**, *1*, 240–245. [CrossRef]
29. Miot-Sertier, C.; Paulin, M.; Dutilh, L.; Ballestra, P.; Albertin, W.; Masneuf-Pomarède, I.; Coulon, J.; Moine, V.; Vallet-Courbin, A.; Maupeu, J.; et al. Assessment of Chitosan Antimicrobial Effect on Wine Microbes. *Int. J. Food Microbiol.* **2022**, *381*, 109907. [CrossRef]
30. Bağder Elmacı, S.; Gülgör, G.; Tokatlı, M.; Erten, H.; İşçi, A.; Özçelik, F. Effectiveness of Chitosan against Wine-Related Microorganisms. *Antonie Van. Leeuwenhoek* **2015**, *107*, 675–686. [CrossRef]
31. Capece, A.; Pietrafesa, R.; Siesto, G.; Romano, P. Biotechnological Approach Based on Selected *Saccharomyces cerevisiae* Starters for Reducing the Use of Sulfur Dioxide in Wine. *Microorganisms* **2020**, *8*, 738. [CrossRef]
32. Siesto, G.; Pietrafesa, R.; Infantino, V.; Thanh, C.; Pappalardo, I.; Romano, P.; Capece, A. In Vitro Study of Probiotic, Antioxidant and Anti-Inflammatory Activities among Indigenous *Saccharomyces cerevisiae* Strains. *Foods* **2022**, *11*, 1342. [CrossRef]
33. Legras, J.L.; Karst, F. Optimisation of Interdelta Analysis for *Saccharomyces cerevisiae* Strain Characterisation. *FEMS Microbiol. Lett.* **2003**, *221*, 249–255. [CrossRef]
34. Capece, A.; Romaniello, R.; Siesto, G.; Romano, P. Diversity of *Saccharomyces cerevisiae* Yeasts Associated to Spontaneously Fermenting Grapes from an Italian "Heroic Vine-Growing Area". *Food Microbiol.* **2012**, *31*, 159–166. [CrossRef] [PubMed]
35. *OIV-MA-AS2-07B*; Chromatic Characteristics. Compendium of International Methods of Analysis. International Organization of Vine and Wine (OIV): Paris, France, 2009.
36. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1999; Volume 299, pp. 152–178.
37. Sánchez-Moreno, C.; Larrauri, J.A.; Saura-Calixto, F. Free Radical Scavenging Capacity of Selected Red, Rosé and White Wines. *J. Sci. Food Agric.* **1999**, *79*, 1301–1304. [CrossRef]
38. Capece, A.; Siesto, G.; Poeta, C.; Pietrafesa, R.; Romano, P. Indigenous Yeast Population from Georgian Aged Wines Produced by Traditional "Kakhetian" Method. *Food Microbiol.* **2013**, *36*, 447–455. [CrossRef] [PubMed]
39. Hammer, Ø.; Harper, D.A.T. Past: Paleontological Statistics Software Package for Educaton and Data Anlysis. *Palaeontol. Electron.* **2001**, *4*, 1.
40. Barbosa, C.; Lage, P.; Esteves, M.; Chambel, L.; Mendes-Faia, A.; Mendes-Ferreira, A. Molecular and Phenotypic Characterization of *Metschnikowia pulcherrima* Strains from Douro Wine Region. *Fermentation* **2018**, *4*, 8. [CrossRef]

41. Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The Effect of Non-*Saccharomyces* Yeasts on Fermentation and Wine Quality. *S. Afr. J. Sci.* **2003**, *24*, 55–62. [CrossRef]
42. Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected Non-*Saccharomyces* Wine Yeasts in Controlled Multistarter Fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, *28*, 873–882. [CrossRef]
43. Picariello, L.; Rinaldi, A.; Blaiotta, G.; Moio, L.; Pirozzi, P.; Gambuti, A. Effectiveness of Chitosan as an Alternative to Sulfites in Red Wine Production. *Eur. Food Res. Technol.* **2020**, *246*, 1795–1804. [CrossRef]
44. Domizio, P.; Romani, C.; Lencioni, L.; Comitini, F.; Gobbi, M.; Mannazzu, I.; Ciani, M. Outlining a Future for Non-*Saccharomyces* Yeasts: Selection of Putative Spoilage Wine Strains to Be Used in Association with *Saccharomyces cerevisiae* for Grape Juice Fermentation. *Int. J. Food Microbiol.* **2011**, *147*, 170–180. [CrossRef]
45. Mendoza, L.M.; Vega-Lopez, G.A.; Fernández de Ullivarri, M.; Raya, R.R. Population and Oenological Characteristics of Non-*Saccharomyces* Yeasts Associated with Grapes of Northwestern Argentina. *Arch. Microbiol.* **2019**, *201*, 235–244. [CrossRef]
46. Mančić, S.; Stamenković Stojanović, S.; Danilović, B.; Djordjević, N.; Malićanin, M.; Lazić, M.; Karabegović, I. Oenological Characterization of Native *Hanseniaspora uvarum* Strains. *Fermentation* **2022**, *8*, 92. [CrossRef]
47. Guzzon, R.; Nardin, T.; Larcher, R. The Controversial Relationship between Chitosan and the Microorganisms Involved in the Production of Fermented Beverages. *Eur. Food Res. Technol.* **2022**, *248*, 751–765. [CrossRef]
48. Stössel, P.; Leuba, J.L. Effect of Chitosan, Chitin and Some Aminosugars on Growth of Various Soilborne Phytopathogenic Fungi. *J. Phytol.* **1984**, *111*, 82–90. [CrossRef]
49. Poznanski, P.; Hameed, A.; Orczyk, W. Chitosan and Chitosan Nanoparticles: Parameters Enhancing Antifungal Activity. *Molecules* **2023**, *28*, 2996. [CrossRef]
50. Omura, Y.; Shigemoto, M.; Akiyama, T.; Saimoto, H.; Shigemasa, Y.; Nakamura, I.; Tsuchido, T. Antimicrobial Activity of Chitosan with Different Degrees of Acetylation and Molecular Weights. *Biocontrol Sci.* **2003**, *8*, 25–30. [CrossRef]
51. Kong, M.; Chen, X.G.; Xing, K.; Park, H.J. Antimicrobial Properties of Chitosan and Mode of Action: A State of the Art Review. *Int. J. Food Microbiol.* **2010**, *144*, 51–63. [CrossRef]
52. Hosseinnejad, M.; Jafari, S.M. Evaluation of Different Factors Affecting Antimicrobial Properties of Chitosan. *Int. J. Biol. Macromol.* **2016**, *85*, 467–475. [CrossRef]
53. Kisko, G.; Sharp, R.; Roller, S. Chitosan Inactivates Spoilage Yeasts but Enhances Survival of *Escherichia coli* O157:H7 in Apple Juice. *J. Appl. Microbiol.* **2005**, *98*, 872–880. [CrossRef]
54. Roller, S.; Covill, N. The Antifungal Properties of Chitosan in Laboratory Media and Apple Juice. *Int. J. Food Microbiol.* **1999**, *47*, 67–77. [CrossRef]
55. Palmeira-de-Oliveira, A.; Passarinha, L.A.; Gaspar, C.; Palmeira-de-Oliveira, R.; Sarmento, B.; Martinez-de-Oliveira, J.; Pina-Vaz, C.; Rodrigues, A.G.; Queiroz, J.A. The Relationship between *Candida* Species Charge Density and Chitosan Activity Evaluated by Ion-Exchange Chromatography. *J. Chromatog. B* **2011**, *879*, 3749–3751. [CrossRef]
56. Seyfarth, F.; Schliemann, S.; Elsner, P.; Hipler, U. Antifungal Effect of High- and Low-Molecular-Weight Chitosan Hydrochloride, Carboxymethyl Chitosan, Chitosan Oligosaccharide and N-Acetyl-d-Glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata*. *Int. J. Pharm.* **2007**, *353*, 139–148. [CrossRef] [PubMed]
57. Liu, H.; Du, Y.; Wang, X.; Sun, L. Chitosan Kills Bacteria through Cell Membrane Damage. *Int. J. Food Microbiol.* **2004**, *95*, 147–155. [CrossRef] [PubMed]
58. Rabea, E.I.; Badawy, M.E.-T.; Stevens, C.V.; Smagghe, G.; Steurbaut, W. Chitosan as Antimicrobial Agent: Applications and Mode of Action. *Biomacromolecules* **2003**, *4*, 1457–1465. [CrossRef] [PubMed]
59. Formosa, C.; Schiavone, M.; Martin-Yken, H.; François, J.M.; Duval, R.E.; Dague, E. Nanoscale Effects of Caspofungin against Two Yeast Species, *Saccharomyces cerevisiae* and *Candida albicans*. *Antimicrob. Agents Chemother.* **2013**, *57*, 3498–3506. [CrossRef]
60. Nguyen, T.H.; Fleet, G.H.; Rogers, P.L. Composition of the Cell Walls of Several Yeast Species. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 206–212. [CrossRef]
61. Castro-Marín, A.; Buglia, A.G.; Riponi, C.; Chinnici, F. Volatile and Fixed Composition of Sulphite-Free White Wines Obtained after Fermentation in the Presence of Chitosan. *LWT* **2018**, *93*, 174–180. [CrossRef]
62. Gómez-Rivas, L.; Escudero-Abarca, B.I.; Aguilar-Uscanga, M.G.; Hayward-Jones, P.M.; Mendoza, P.; Ramírez, M. Selective Antimicrobial Action of Chitosan against Spoilage Yeasts in Mixed Culture Fermentations. *J. Ind. Microbiol. Biotechnol.* **2004**, *31*, 16–22. [CrossRef]
63. Sonni, F.; Cejudo Bastante, M.J.; Chinnici, F.; Natali, N.; Riponi, C. Replacement of Sulfur Dioxide by Lysozyme and Oenological Tannins during Fermentation: Influence on Volatile Composition of White Wines. *J. Sci. Food Agric.* **2009**, *89*, 688–696. [CrossRef]
64. Herraiz, T.; Martin-Alvarez, P.J.; Reglero, G.; Herraiz, M.; Cabezudo, M.D. Differences between Wines Fermented with and without Sulphur Dioxide Using Various Selected Yeasts. *J. Sci. Food Agric.* **1989**, *49*, 249–258. [CrossRef]
65. Spagna, G.; Barbagallo, R.N.; Pifferi, P.G. Fining Treatments of White Wines by Means of Polymeric Adjuvants for Their Stabilization against Browning. *J. Agric. Food Chem.* **2000**, *48*, 4619–4627. [CrossRef]
66. Filipe-Ribeiro, L.; Cosme, F.; Nunes, F.M. Reducing the Negative Sensory Impact of Volatile Phenols in Red Wine with Different Chitosans: Effect of Structure on Efficiency. *Food Chem.* **2018**, *242*, 591–600. [CrossRef] [PubMed]

67. Popa, M.-I.; Aelenei, N.; Popa, V.I.; Andrei, D. Study of the Interactions between Polyphenolic Compounds and Chitosan. *React. Funct. Polym.* **2000**, *45*, 35–43. [CrossRef]
68. Mine Kurtbay, H.; Bekçi, Z.; Merdivan, M.; Yurdakoç, K. Reduction of Ochratoxin A Levels in Red Wine by Bentonite, Modified Bentonites, and Chitosan. *J. Agric. Food Chem.* **2008**, *56*, 2541–2545. [CrossRef] [PubMed]