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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: The use of chitosan as antimicrobial agent in different agri-food application is widely reported; regarding the wine sector, the OIV (International Organization of Vine and Wine) 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.

This work investigated the antimicrobial efficiency of chitosan from two different sources, which were commercial (crustaceans) and insect (*Hermetia illucens*), against non-*Saccharomyces* yeasts involved in grape must fermentation, such as *Metschnikowia pulcherrima*, *Candida zemplinina*, *Hanseniaspora uvarum*, *H. guilliermondii*, *H. osmophila*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii*, *Lachancea thermotolerans*, *Pichia kluyveri*, *P. kudriavzevii*, *P. anomala*. Results show that the tested strains exhibited medium/high resistance to the chitosan, but in some cases the behaviour varied in function of species/strain. As regards the different chitosan source, only few strains exhibit different resistance levels depending on the chitosan type used. Treatment with two chitosan types during grape must fermentation inoculated with *S. cerevisiae*, showed lower antimicrobial activity than the SO_2, but the combined use with sulphur dioxide showed better effect than the use of chitosan alone for both of them.

The evaluation of suitability of chitosan obtained from a sustainable source, such as insects, will allow to give new information for future application of this natural compound for the production of wine with low sulphite content.

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

Keywords: chitosan, non-*Saccharomyces* wine yeasts, antimicrobial activity, wine fermentation, insect-based chitosan.

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 those undesirable, such as yeasts, lactic 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 increased the market request toward the production of wines with reduced sulphite content. Indeed, wine quality is compromised with 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].

As regards the problems related to human health, adverse reactions in gastrointestinal tract, on the skin and on respiratory system can occur in "sulphite-sensitive" population after sulphites ingestion [4,5,6]. For these reasons, considering the large use of SO_2 in different food products, the European Community (EU) established limit doses of this compound in foods as the risk is correlated to an 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, also, limits on the use of this antimicrobial have been established. Indeed, the maximum dose allowed of total SO_2 content in red wine is 150 mg/L and in white and rosé wine 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 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 deacetylation process 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 removing the acetyl groups, allows to obtain a compound more soluble and suitable 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 concentration of unwanted microorganisms, especially *Brettanomyces* spp.

In addition to the already authorised purposes, several studies also showed other applications of chitosan, for example as 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 polysaccharide since they contain from 15 to 40% of chitin in their exoskeleton [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 potential risk of allergic reactions [18]. Regarding insect-based chitosan, to date, the U.S. The Food and Drug Administration (US FDA) doesn't include insects as a major food allergen [19]. Insects can be considered an alternative and

sustainable source to obtain chitosan, with numerous advantages, such as the reproducibility of insect rearing conditions that allow to obtain the chitin during the whole year [20].

Some insects have also the advantage of bioconverting waste products, as they can feed on different organic substrates [21]. Among these, great interest is being aroused by 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, the chitosan is used in oenology mainly to control the wine-contaminating yeast *Brettanomyces bruxellensis*. Indeed, several studies 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 about 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 was addressed to explore the potentiality to use chitosan of two different sources (commercial and insect-based) against non-*Saccharomyces* yeasts involved in the first steps of grape must fermentation, with the final aim to produce wine with low sulphite content. The evaluation of suitability of chitosan obtained from an unconventional source, such as insects, will provide new information for future application of this natural compound as antimicrobial compound for the production of wine with low sulphite content and for other food processing.

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 chosen strains, belonging to UNIBAS Yeast Collection (UBYC), University of Basilicata (Italy), are reported in Table 1.

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

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

Species	Strain code	Origin
Metschnikowia pulcherrima	AII-136	Bees
	4-11; 4R1	Grapes
Lachancea thermotolerans	AII-134	Bees
	4-14	Grapes
Pichia kluyveri	AII-110	Bees
Pichia kudriavzevii	AII-177	Bees
	4-16	Grapes
Pichia anomala	AII-186	Bees
Candida zemplinina	TSE	Grapes
	FCB6	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

2.2. Evaluation of SO_2 and chitosan tolerance during inoculated fermentations

The twenty strains were tested for tolerance to SO_2 and commercial chitosan, on the basis of the effect of the two antimicrobial substances on viability of these strains during grape must fermentation. For both the antimicrobials, stock solutions (10 g/L) were prepared. 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, in order to obtain the complete dissolution of chitosan, and it 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 of sugar content). 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 one of them 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 added with 50 mg/L of SO_2, the amount frequently used during cellar fermentations [31]; (b) grape must added with 100 mg/L of commercial chitosan (Merck KGaA), the amount authorized by the OIV [15] to control the spoilage microorganisms in wine; (c) grape must added with 20 mg/L of SO_2 and 100 mg/L of commercial chitosan, in order to try to reduce the amounts 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 in the first steps of the fermentation. At this aim, the viability of inoculated starters was checked by microbial viable count at T_0 and T_{48}, using WL agar medium. The dilution plates containing a statistically representative number of colonies were counted.

For each antimicrobial treatment, the strain resistance percentage was calculated through 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 by 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, whereas 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

The twenty non-*Saccharomyces* yeasts were tested for determination of resistance level of two types of chitosan, the commercial from shrimp shells purchased from Merck KGaA (Darmstadt, Hesse, Germany) and insect-based chitosan extracted from *Hermetia illucens* pupal exuviae. The raw samples were provided by Xflies s.r.l (Potenza, Italy). Appropriate volumes from the stock solutions (10 g/L) of both chitosan types solubilized in acetic acid 1% v/v, were added to an agarized medium, composed by pasteurized grape must (Aglianico del Vulture) added with aqueous agar solution (4% agar), to obtain a range of concentrations of 100, 200, 300, 400 mg/L. The yeast strains were inoculated at the initial concentration of approximately 1×10^6 cells/mL and the plates were incubated for 48 h. Chitosan-free medium, inoculated with test strains, was used as a control. For each strain, the resistance level was expressed as the maximum dose at which microbial growth was observed. All experiments were conducted in duplicates.

2.4. Use of antimicrobial treatments during inoculated fermentations at laboratory scale

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

The fermentations were performed in flasks containing 2L of fresh Aglianico del Vulture grape must and skins (255.8 g/L of sugar content), gently supplied by the Cantina di Venosa cellar (Basilicata, Italy) during the 2023 vintage. The grape must was added with 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 treatments, the flasks were inoculated with 4LBI-3 *Saccharomyces cerevisiae* strain (inoculum level at 6×10^6 cells/mL). This was a selected indigenous strain isolated from "Aglianico del Vulture" grape must and belonging to the UBYC collection [32]. The fermentations were conducted in duplicate, and the fermentation temperature was kept at 20°C.

The fermentation kinetic was daily monitored by measuring the sugar consumption. The main wine chemical parameters (sugar content, ethanol production, total acidity, volatile acidity, pH, malic acid) were measured daily via Fourier Transform Infrared WineScan instrument (OenoFoss™, Hillerød, Denmark). The evolution of yeast population was monitored at different times (1, 2, 4, 7, 10, 17 days of fermentation) by viable plate count using two media, WL Nutrient Agar medium (Oxoid, Hampshire, UK), a differential media 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 5 days, after that the dilution plates statistically representative were counted. The colonies showing *S. cerevisiae* morphology were submitted to amplification of interdelta region with δ2/δ12 primer pair [33], following a previously described protocol [34], in order to check the dominance level of inoculated starter.

2.5. Wine analysis

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

Chemical analysis

The chemical wine parameters (sugar content, ethanol production, total and volatile acidity, pH, malic acid) were measured via Fourier Transform Infrared WineScan instrument (OenoFoss™, Hillerød, Denmark).

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 1 mL wine sample was measured using spectrophotometer Spectrostar^{nano} (BMG LABTECH, Ortenberg, Germany) to evaluate the principal chromatic characteristics of wine, which are the intensity and the hue. The intensity was calculated as the sum of OD_{420}, OD_{520}, OD_{620} and the hue as the ratio between OD_{420} and OD_{520}.

Polyphenols content detection

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

Antioxidant activity

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

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

Aromatic compounds

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

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

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) were 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 was evaluated during inoculated fermentations, by determining the influence of these antimicrobials on cell viability of yeast strains in the first step of fermentation inoculated with each strain.

The effect of antimicrobial treatments was reported in Table 2, where the resistance percentage was based on the ratio between generation number of treated and control samples in the first 48 h of fermentations. Generally, the tested strains were more resistant to chitosan than SO_2, except for *M. pulcherrima* (in particular 4-11 and 4R1) and *T. delbrueckii* strains. For some strains, no differences statistically significant among the three treatments were found, such as AII-136 (*M. pulcherrima*), TSE (*C. zemplinina*) and AII-186 (*P. anomala*). As regards the use of the combined treatment involving the use of chitosan (100 mg/L) and a reduced amount of SO_2 (20 mg/L), the resistance percentage was similar to those observed for the single treatment, mainly for the chitosan addition. Only in few strains, statistically significant differences were found 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 to the species but also to the single strain of the analyzed yeasts. One *M. pulcherrima* strain (AII-136) is highly sensitive to all the antimicrobials, whereas two strains (4-11 and 4R1) were low tolerant to chitosan and high resistant to SO_2. The ability of chitosan treatment to affect *M. pulcherrima* was reported also by other authors [29]. Otherwise, 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 the resistance was evaluated in YPD agar medium (with pH adjusted to 3.5), while it's reported that the efficacy

of chitosan decreased under winemaking conditions. The high SO_2-resistance of *M. pulcherrima* was reported by other studies, using different strains and methodologies [40–42]. All the *Hanseniaspora* strains tested in our study were unaffected by chitosan addition, in agreement with previous findings reporting that chitosan treatment was not able to prevent the development of *H. uvarum* [29,43]. Particularly, 1P3 *H. uvarum* strain exhibited a very high tolerance to both chitosan and SO_2. Other studies reported the high variability for SO_2 tolerance among *Hanseniaspora* yeasts [44,45] and strains characterized by tolerance at concentrations much higher than those usually used in the winemaking were described [46].

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

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

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.

3.2. Evaluation of strain-resistance to commercial and insect-based chitosan

Figure 1 illustrates the resistance levels, corresponding to the highest tolerated dose (reported as mg/L) by twenty non-*Saccharomyces* yeast strains when treated with commercial and insect-based chitosan. Most noticeable, very few studies were addressed to evaluate the resistance level to chitosan among yeast strains belonging to different yeast species frequently encountered during spontaneous wine fermentation, such as *C. zemplinina*, *T. delbrueckii*, *L. thermotolerans* [29,47]. Furthermore, the antimicrobial activity of an

innovative and sustainable source of chitosan, which was insect-based chitosan, was firstly evaluated in this research.

As reported in figure 1, most yeast strains exhibited similar behaviour with 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*), 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 chitosan type used. The lowest resistance levels were observed in strains AII-136, 4-11 and 4R1 (*M. pulcherrima*), 2R9 (*H. guilliermondii*), 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, observing a higher resistance to the commercial chitosan compared to the insect-based one, showing a better efficacy of the latter. The opposite behaviour, however, occurred in strain AP1 (*H. uvarum*) and TM5-2 (*H. guilliermondii*), where commercial chitosan had better antimicrobial action than insect-based one.

The data suggest that insect-based chitosan generally performs on par with commercial chitosan across a wide range of yeast strains, whereas only few strains exhibit different resistance levels depending on the chitosan type used. The antimicrobial activity of the chitosan is correlated to some chemical characteristics, such as deacetylation degree and molecular weight, with stronger activity for higher deacetylation degree and low molecular weight and being under acidic conditions for its amino groups to be charged [48,49]. By comparing the chemical characteristics of the two chitosan types, we would have expected a higher antimicrobial activity of insect-based chitosan (deacetylation degree > 90%, molecular weight 80-100 kDa) [14] than commercial one (deacetylation degree > 75%, molecular weight 190-375 kDa). However, other factors, among of which the type of microorganisms, can affect its antimicrobial activity and to achieve the highest antimicrobial activity, optimum conditions of chitosan application should be investigated and tested before its application [50].

As regards behaviour among strains of some species, all *M. pulcherrima* showed the same resistance level, which were 200 mg/L, independently from the chitosan source. The low tolerance of *M. pulcherrima* was already reported in chitosan treatment of apple juice [51], but no further data on the chitosan tolerance of this species are available in literature. Strains of other species, such as *Z. bailii* (CR-1 and CR-2) and *C. zemplinina* (TSE and FCB6), showed a resistance level which was strain-dependant. The resistance level of CR-1 strain (300 and 200 mg/L for commercial and insect-based chitosan, respectively) was lower than CR-2 (400 and 300 mg/L for commercial and insect-based chitosan, respectively). Our findings are in accordance with other works [30,52], reporting that *Z. bailii* was susceptible to chitosan at 0.1 and 0.4 g/L. As previously reported [47], the yeasts having high fermentative attitude, such as *T. delbrueckii*, showed a remarkable resistance to chitosan. However, it has been found that chitosan may be an effective fungicidal, but different studies report that rather high concentrations are required to inhibit yeast growth [53–56].

Overall, these results indicated a 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's well known that cell surface properties, cell wall composition, such as polysaccharide content, and molecular organization of the cell wall varied among different yeast species and strains [57,58].

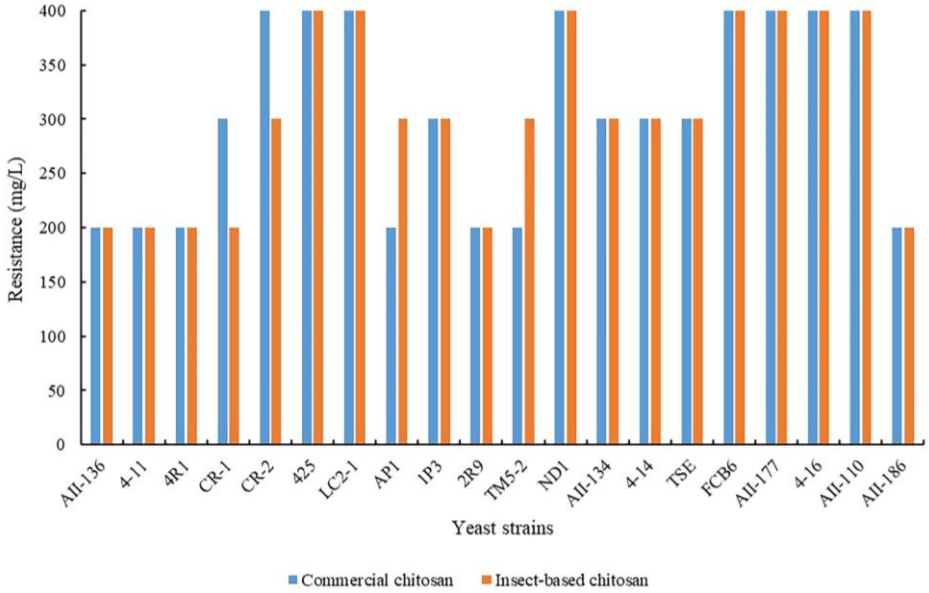

Figure 1. Non-*Saccharomyces* resistance level to commercial and insect-based chitosan performed on agarized grape must.

3.3. Use of antimicrobial treatments during inoculated fermentations at laboratory scale

This step was addressed to evaluate the efficiency of different antimicrobial treatments during laboratory-scale fermentations, 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 level (20 mg/L).

Fermentation kinetics were monitored by measuring sugar consumption and ethanol production (Figure 2). In general, all the fermentations were completed and lasted seventeen days. The final sugar level was less than 1 g/L and ethanol approximately 15 % v/v for all the wines. The fermentation added with 50 mg/L SO_2 showed a different trend in comparison to the others during the first days of the process, with slower sugar consumption and ethanol production. Opposite results were reported by other authors, which found slower fermentation rates in grapes treated with chitosan [43,59].

However, by the fourth day of fermentation, the differences were reduced, and the fermentation trend was similar for all the conditions. In all cases, the alcoholic fermentations were completed regardless of chitosan and/or SO_2 addition. However, as previously reported [30] chitosan seemed not to alter the fermentative performance of *Saccharomyces cerevisiae* starter, although some authors [47] reported the lengthening of the lag phase due to the initial killing of a part of the yeast population, demonstrating its potential applicability in wine fermentation.

To investigate the antimicrobial effect of all the treatments, the evaluation of yeast population dynamics during the different fermentations was carried out by viable plate count after 1, 2, 4, 7, 10, 17 days of fermentation. In general, the trend was similar for all the fermentations, showing a decrease of non-*Saccharomyces* cells viability during time; however, after 7 days of fermentation, no colonies of non-*Saccharomyces* yeasts were found for all the conditions. The viable count of non-*Saccharomyces* yeasts, reported in figure 3A, was affected by the treatment. On day 1, after 24 hours from the starter inoculum and antimicrobials addiction, the lowest level of viable non-*Saccharomyces* cells was found in the fermentation added with only sulphur dioxide, while the highest level was detected in the fermentation added 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 justify the lowest initial rate of fermentation observed in this sample in comparison to other antimicrobial treatments (Figure 2).

On day 2, after 48 hours, the fermentation with the use of SO_2 alone showed again the lowest level of viable non-*Saccharomyces* cells, but this value was slightly higher than day 1, probably due to an adaptation of the yeasts to the medium. Furthermore, as regards the chitosan, both the types showed lower activity than SO_2, but the combined use with sulphur dioxide showed better effect than the use of chitosan alone for both of them. 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 the reduction of viable cell number below the detection limit was observed on the fourth day of fermentation, whereas in our study it happened on the 7-fermentation day. Other authors [43] reported lower inhibition 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 about 50% (Figure 2), a reduction of viable cells was observed only for SO_2 and commercial chitosan treatments, whereas for the other fermentations the number of viable cells was very similar to those observed at the second fermentation day.

Lastly, on day 7, the highest reduction in the viable cells was observed for all the trials, and this result can be related also to the ethanol content reached at this point, that was around 9 % (v/v) for almost all the fermentations (Figure 2). Also in this step, the highest reduction was showed 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 levels 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, which found a drastic decline of 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 6 days, when chitosan was applied in combination with SO_2.

Regarding the evolution of *S. cerevisiae* population (Figure 3B), the trend was similar in all fermentations and the number of viable cells did not show difference among the treatments with the different antimicrobial compounds. This may mean that neither sulphur dioxide nor commercial or insect-based chitosan interferes with the normal development of *S. cerevisiae*. 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 4LBI-3 strain during overall the process (data not shown).

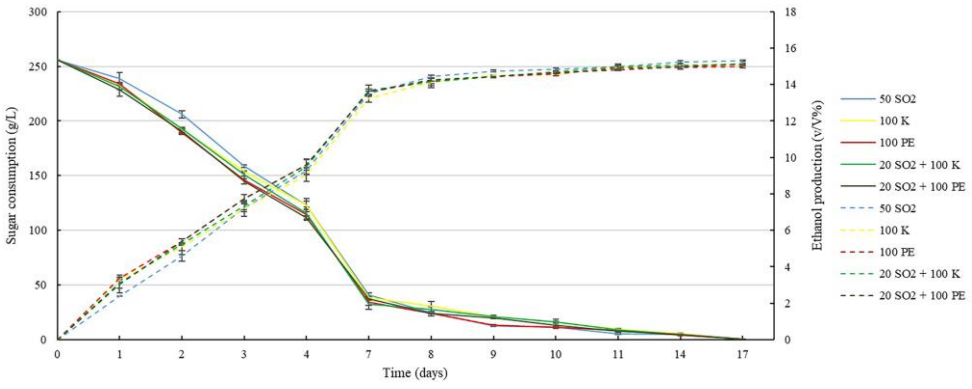

Figure 2. Sugar consumption (solid line) and ethanol production (dashed lines) during inoculated fermentations at laboratory scale added with different antimicrobials: 50 mg/L of SO_2 (50 SO_2); 100 mg/L of commercial chitosan (100 K); 100 mg/L of insect-based chitosan (100 PE); 20 mg/L of SO_2 and 100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

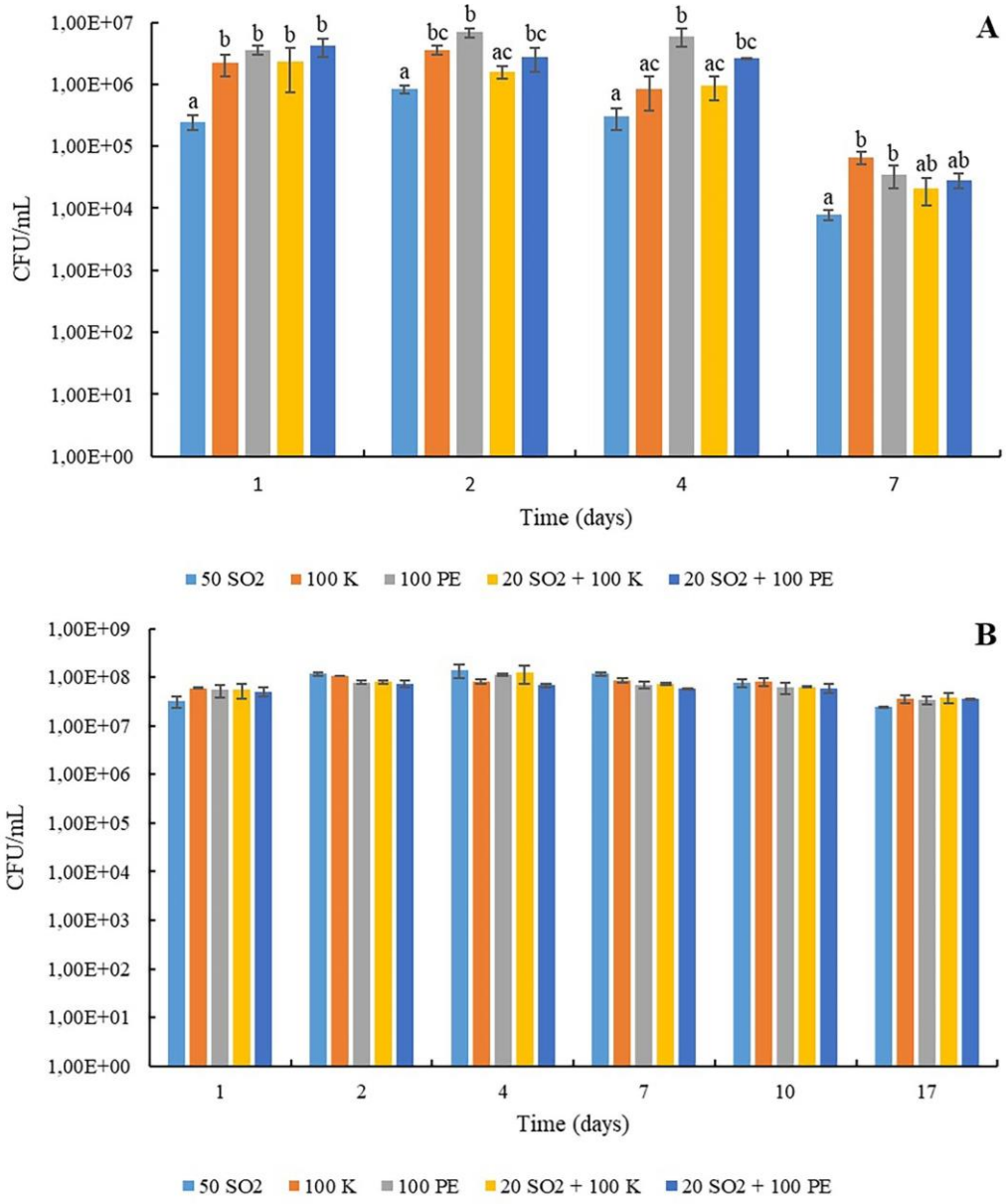

Figure 3. Viable cell population of non-*Saccharomyces* (A) and *S. cerevisiae* (B) yeasts detected at different times of fermentation inoculated with *Saccharomyces cerevisiae* 4LBI-3 and added with different antimicrobials: 50 mg/L of SO_2 (50 SO_2); 100 mg/L of commercial chitosan (100 K); 100 mg/L 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

3.4. Analyses of experimental wines obtained with different antimicrobial treatments

The general oenological parameters and main volatile compounds detected in the experimental wines obtained with different antimicrobial treatments are reported in Table 3. As regards the alcohol content, similar values (ranging between 14.98 and 15.31 % v/v) were found in all the wines, with the lowest level detected in the wine obtained by grape must added with insect-based chitosan. Otherwise, the highest ethanol level was found in wine produced with SO_2 addition, which was the wine with the lowest sugar residual content. No differences were found among the wines for total acidity, whereas the volatile acidity varied among the different treatments, although in all the wines the values were

in 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's well known that the non-*Saccharomyces* yeast species prevalent in the first steps of fermentations (such as *Hanseniaspora uvarum/Kloeckera apiculata*) are generally known as a high producer of volatile acidity [41]. Regarding the content of main volatile compounds affecting wine aroma, despite different treatments, the wines showed no significant differences in level of acetaldehyde, n-butanol and acetoin. For the other analyzed compounds, statistical differences were found. In particular, the wine treated with the only SO_2 showed the lowest level of ethyl acetate and n-propanol compared to all the others. As regards 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 differences statistically significant compared to wine obtained with 50 mg/L of sulphur dioxide. Similar behaviour was observed for 2-methyl-1-butanol. Other authors [59] found higher level of 3-methyl-1-butanol in SO_2 added wines than chitosan treated samples, confirming previous finding postulating that SO_2 presence during fermentation favours a rapid consumption of amino acids [60,61].

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, total polyphenols as mg GAE/L.

The experimental wines were also analyzed for the content of total polyphenols and antioxidant activity, to evaluate the influence of the used antimicrobials on these parameters (Table 3). As regards the polyphenol content measured by the Folin-Ciocalteu reaction, a certain variability was observed among the experimental wines. The sample treated with 50 mg/L of SO_2, showed the highest polyphenols 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 and 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 polyphenols content, not very different from each other, ranging from 1703 and 1790 mg GAE/L.

Similar trend was observed for the evaluation of the antioxidant activity, calculated as % of DPPH reduction. The highest level was showed in experimental wine produced with SO_2 alone and as found for the polyphenol content, the lowest value was obtained in samples treated with both chitosans alone.

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

This result might be correlated with the high affinity to phenolic compounds of chitosan in wine, reported by other authors [62]. Indeed, in the wine, in consequence of its low pH, chitosan is a polymer with a high positive charge density, and this condition allows the formation of efficient complex (by means of non-covalent force, such as hydrophobic, electrostatic interactions and/or hydrogen bonding) between chitosan and polyphenols and the formation of soluble complexes that can aggregate, with subsequent precipitation [63,64].

The chromatic characteristics of the experimental wines were reported in Figure 4; the colour intensity was calculated as the sum of OD_{420}, OD_{520}, OD_{620} and the hue as the ratio between OD_{420} and OD_{520}. As regards colour intensity, differences statistically significant were found among the different treatments. Wines from treatment with chitosan showed higher intensity than wine from SO_2-added, for commercial chitosan. The lowest colour intensity was observed for the wine treated with combined use of SO_2 and commercial chitosan, whereas the use of insect-based chitosan+SO_2 gave wine with colour intensity similar to those of wine obtained from insect-based chitosan alone Some studies reported that chitosan appreciably reduces colour intensity, but the reduction is dependent on the doses of chitosan applied [65].

As regards the hue, the lowest value was found in the wine obtained with the use of 50 mg/L of SO_2, while the wine from combined use of SO_2 and commercial chitosan showed the highest value. Among the other wines, no differences statistically significant were observed.

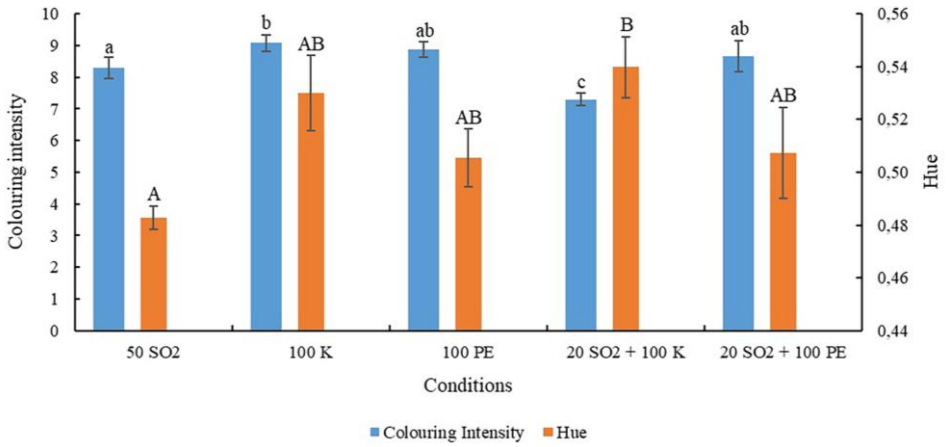

Figure 4. Colour intensity and hue of wine produced with the use of different antimicrobial treatments: 50 mg/L of SO_2 (50 SO_2); 100 mg/L of commercial chitosan (100 K); 100 mg/L of insect-based chitosan (100 PE); 20 mg/L of SO_2 and 100 mg/L of commercial chitosan (20 SO_2 + 100 K); 20 mg/L of SO_2 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. Lower case letters indicate differences in colour intensity and capital letters indicate differences in hue.

4. Conclusions

In this work, the effect of two chitosan types, commercial one and insect-based chitosan, on non-*Saccharomyces* yeasts associated with grape must fermentation was evaluated. The overall results demonstrated that the wine yeasts tested in this study showed medium/high resistance to the chitosan, but a variable behaviour in function of strain or species was observed. Insect-based chitosan generally performs on par with commercial chitosan across a wide range of yeast strains, whereas only few strains exhibit different resistance levels depending on the chitosan type used.
The addition of the two chitosan types during grape must fermentation inoculated with *S. cerevisiae*, showed lower antimicrobial activity than the SO_2, but the combined use with sulphur dioxide showed better effect than the use of chitosan alone for both of them. However, chitosan treatment does not seem to inhibit *Saccharomyces cerevisiae*, confirming the potentiality for the 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 the chitosan, for insect-based chitosan. The evaluation of suitability of chitosan obtained from a sustainability source, such as insects, will allow to give new information for future application of this natural compound to produce wine with low sulphite content and for other food processing.

Author Contributions: FT: Conceptualization; Data curation; Investigation; Methodology; Writing - original draft. RP: Data curation; Formal analysis; Investigation; Methodology. GS: Data curation; Methodology; Writing - review & editing. CS: Writing - review & editing; RS: Writing - review & editing; PF: Writing - review & editing; AC: Conceptualization; Project administration; Resources; Supervision; Writing - original draft; and Writing - review & editing.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data Availability Statement: Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

Acknowledgments: FT received a doctoral fellowship supported by the Regione Basilicata Government. GS received a grant from European Commission – NextGenerationEU for her position through the Project "Strengthening the MIRRI Italian Research Infrastructure for Sustainable

Bioscience and Bioeconomy" (code n. IR0000005). The authors would like to thank the "Cantina di Venosa" cellar that kindly supplied grape must samples.

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, doi:10.19040/ecocycles.v6i2.181.
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, doi:10.1016/j.tifs.2014.11.004.
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, doi:10.3390/molecules27113535.
4. Vally, H.; Misso, N.L.A.; Madan, V. Clinical Effects of Sulphite Additives. *Clinical & Experimental Allergy* **2009**, *39*, 1643–1651, doi:10.1111/j.1365-2222.2009.03362.x.
5. Vally, H.; Misso, N. LA 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, doi:10.9734/EJNFS/2015/11557.
7. JECFA, J.F.A.O. Safety Evaluation of Certain Food Additives and Contaminants. In Proceedings of the Prepared by the Seventy-Third Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA); 2006.
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, doi:10.1111/1541-4337.12422.
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, doi:10.3390/beverages8030058.
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, doi:10.1080/10408398.2020.1798871.
11. Lárez Velásquez, C. Chitosan and Its Applications in Oenology. *OENO One* **2023**, *57*, 121–132, doi:10.20870/oeno-one.2023.57.1.7262.
12. Pellis, A.; Guebitz, G.M.; Nyanhongo, G.S. Chitosan: Sources, Processing and Modification Techniques. *Gels* **2022**, *8*, 393, doi:10.3390/gels8070393.
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; 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, doi:10.1038/s41598-022-10423-5.
15. International Organization of Vine and Wine (OIV) Wines-Treatment Using Chitosan. In *OIV-ENO-338a-2009*; International code of oenological practices., Ed.; 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, doi:10.1021/jf5025664.
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, doi:10.1016/j.jclepro.2017.01.082.
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. *Annals of Allergy, Asthma & Immunology* **2016**, *116*, 462–463, doi:10.1016/j.anai.2016.02.004.
19. de Gier, S.; Verhoeckx, K. Insect (Food) Allergy and Allergens. *Mol Immunol* **2018**, *100*, 82–106, doi:10.1016/j.molimm.2018.03.015.
20. van Huis, A. Potential of Insects as Food and Feed in Assuring Food Security. *Annu Rev Entomol* **2013**, *58*, 563–583, doi:10.1146/annurev-ento-120811-153704.
21. Scieuzo, C.; Franco, A.; Salvia, R.; Triunfo, M.; Addeo, N.F.; Vozzo, S.; Piccolo, G.; Bovera, F.; Ritieni, A.; Francia, A. Di; et al. Enhancement of Fruit Byproducts through Bioconversion by *Hermetia Illucens* (Diptera: Stratiomyidae). *Insect Sci* **2023**, *30*, 991–1010, doi:10.1111/1744-7917.13155.
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, doi:10.1038/s41598-022-12150-3.
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, doi:10.1111/jam.12682.
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. *European Food Research and Technology* **2014**, *238*, 641–652, doi:10.1007/s00217-013-2143-2.

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, doi:10.20870/oeno-one.2016.50.1.54.
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 Studies* **2014**, *1*, doi:10.4081/ws.4574.
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*, doi:10.3389/fmicb.2020.571067.
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*. *Journal of Chitin and Chitosan Science* **2013**, *1*, 240–245, doi:10.1166/jcc.2013.1037.
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, doi:10.1016/j.ijfoodmicro.2022.109907.
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, doi:10.1007/s10482-014-0362-6.
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*, doi:10.3390/microorganisms8050738.
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, doi:10.3390/foods11091342.
33. Legras, J.-L.; Karst, F. Optimisation of Interdelta Analysis for *Saccharomyces Cerevisiae* Strain Characterisation. *FEMS Microbiol Lett* **2003**, *221*, 249–255, doi:10.1016/S0378-1097(03)00205-2.
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, doi:10.1016/j.fm.2012.03.010.
35. International Organization of Vine and Wine (OIV) Chromatic Characteristics. In *OIV-MA-AS2-07B*; Compendium of international methods of analysis, Ed.; 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*; 1999; Vol. 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, doi:10.1002/(SICI)1097-0010(19990715)79:10<1301::AID-JSFA367>3.0.CO;2-Y.
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, doi:10.1016/j.fm.2013.07.008.
39. Hammer, Ø.; Harper, D.A.T. Past: Paleontological Statistics Software Package for Educaton and Data Anlysis. *Palaeontologia electronica* **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, doi:10.3390/fermentation4010008.
41. Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The Effect of Non-Saccharomyces Yeasts on Fermentation and Wine Quality. *South African Journal of Enology and Viticulture* **2003**, *24*, doi:10.21548/24-2-2638.
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, doi:10.1016/j.fm.2010.12.001.
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. *European Food Research and Technology* **2020**, *246*, 1795–1804, doi:10.1007/s00217-020-03533-9.
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, doi:10.1016/j.ijfoodmicro.2011.03.020.
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, doi:10.1007/s00203-018-1601-4.
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, doi:10.3390/fermentation8030092.
47. Guzzon, R.; Nardin, T.; Larcher, R. The Controversial Relationship between Chitosan and the Microorganisms Involved in the Production of Fermented Beverages. *European Food Research and Technology* **2022**, *248*, 751–765, doi:10.1007/s00217-021-03919-3.
48. Stössel, P.; Leuba, J.L. Effect of Chitosan, Chitin and Some Aminosugars on Growth of Various Soilborne Phytopathogenic Fungi. *Journal of Phytopathology* **1984**, *111*, 82–90, doi:10.1111/j.1439-0434.1984.tb04244.x.
49. Poznanski, P.; Hameed, A.; Orczyk, W. Chitosan and Chitosan Nanoparticles: Parameters Enhancing Antifungal Activity. *Molecules* **2023**, *28*, 2996, doi:10.3390/molecules28072996.
50. Hosseinnejad, M.; Jafari, S.M. Evaluation of Different Factors Affecting Antimicrobial Properties of Chitosan. *Int J Biol Macromol* **2016**, *85*, 467–475, doi:10.1016/j.ijbiomac.2016.01.022.

51. 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, doi:10.1111/j.1365-2672.2004.02527.x.
52. Roller, S.; Covill, N. The Antifungal Properties of Chitosan in Laboratory Media and Apple Juice. *Int J Food Microbiol* **1999**, *47*, 67–77, doi:10.1016/S0168-1605(99)00006-9.
53. 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. *Journal of Chromatography B* **2011**, *879*, 3749–3751, doi:10.1016/j.jchromb.2011.07.034.
54. 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**, doi:10.1016/j.ijpharm.2007.11.029.
55. Liu, H.; Du, Y.; Wang, X.; Sun, L. Chitosan Kills Bacteria through Cell Membrane Damage. *Int J Food Microbiol* **2004**, *95*, 147–155, doi:10.1016/j.ijfoodmicro.2004.01.022.
56. 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, doi:10.1021/bm034130m.
57. 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, doi:10.1128/AAC.00105-13.
58. 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, doi:10.1007/s002530051278.
59. 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, doi:10.1016/j.lwt.2018.03.003.
60. 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, doi:10.1002/jsfa.3503.
61. 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, doi:10.1002/jsfa.2740490213.
62. 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, doi:10.1021/jf0005519.
63. 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, doi:10.1016/j.foodchem.2017.09.099.
64. 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, doi:10.1016/S1381-5148(00)00009-2.
65. 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, doi:10.1021/jf073419i.