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Assessment of chitosan antimicrobial effect on wine microbes

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ABSTRACT

Chitosan is an active highly charged polysaccharide that has initially been developed in oenology to eliminate the spoilage yeast *B. bruxellensis*. However, different forms of chitosan exist, some complying with EU regulation for their use in wines, others not. Moreover, with the trend in oenology of limiting SO_2, more and more questions arise as to the impact of chitosan on other microorganisms of the grape and wine environment.

We investigated the antimicrobial efficiency of chitosan on a large oenological microbial collection, englobing technological as well as spoilage microorganisms. Results show that most species are affected at least transiently. Furthermore, a high variability prevails within most species and sensitive, intermediate and tolerant strains can be observed. This study also highlights different efficiencies depending on the wine parameters or the winemaking stage, giving important indications on which winemaking issues can be solved using chitosan.

Chitosan treatment does not seem to be appropriate to limit the musts microbial pressure and *Saccharomyces cerevisiae* cannot be stopped during alcoholic fermentation, especially in sweet wines. Likewise, acetic acid bacteria are poorly impacted by chitosan. After alcoholic fermentation, chitosan can efficiently limit non-*Saccharomyces* yeast and lactic acid bacteria but special care should be given as to whether malolactic fermentation is wanted or not. Indeed, *O. oeni* can be severely impacted by chitosan, even months after treatment.

Finally, this study highlights the crucial importance of the chitosan type used in its efficiency towards microbial stabilization. While a high molecular weight chitosan has limited antimicrobial properties, a chitosan with a much lower one, complying with EU and OIV regulation and specifications for its use in wine is much more efficient.

1. Introduction

Many flaws may affect wine quality and merchantability (Grainger, 2021). As a consequence, wines can no longer be suitable for consumption while others see their typicity or ageing potential severely affected. Several wine faults have a microbiological origin: they may be due to fermentation failure/delay, or linked to the development of spoilage microorganisms.

The main species responsible for alcoholic fermentation is *Saccharomyces cerevisiae*. However, many studies mention that non-*Saccharomyces* (NS) yeast can have beneficial effects on wine, contributing to its organoleptic complexity. Several species have thus been proposed for co-fermentation in association with *S. cerevisiae* (Albertin et al., 2014a, 2014b; Capozzi et al., 2015; Englezos et al., 2019; Knight et al., 2015; Masneuf-Pomarede et al., 2016). Furthermore, a growing number of winemakers take advantage of the various natural NS yeast present on the grapes and in the winery, introducing maceration steps before fermentations, in order to allow these species to develop and impact the wine (Ciani et al., 2010; Gianvito et al., 2022; Goode and Harrop, 2013). However, some NS yeast species are known to produce off flavours, contributing to wine spoilage (Grainger, 2021). In ageing wines, one the most feared microbial spoilage in red wines is volatile phenols production, associated with the development of the yeast *Brettanomyces bruxellensis* (Agnolucci et al., 2017; Cibrario et al., 2020; Harrouard et al.,

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2022; Oro et al., 2019; Romano et al., 2009). The presence of acetic acid bacteria (AAB) or of specific lactic acid bacteria (LAB) species or strains also increases the risk of microbial spoilage as they may increase volatile acidity, induce wine mousiness, synthetize biogenic amines, or form beta glucan leading to ropiness (Bartowsky, 2009; Dimopoulou and Dols-Lafargue, 2021; Lonvaud-Funel, 2001; Pelonnier-Magimel et al., 2020; Visciano and Schirone, 2022). In other cases, film forming yeasts may grow on the surface of wines and others can induce undesirable sweet wines re-fermentation (Escott et al., 2017).

As a result, extreme care must be taken during the pre-maceration and fermentation steps but also all along wine ageing to limit microbial spoilage. The most common way to prevent or eliminate unwanted microbes is sulphur dioxide (SO_2) addition. However, as risks of sulphite intolerance and even acute allergy exist, the European Union has classified SO_2 as a priority food allergen (EU Regulation No. 1169/2011, Annex II). Wine bottles labelling became mandatory (Regulation EU 1169/2011). The reduction of sulphites is also considered by consumers as a guarantee of the "natural" character of the wine (Goode and Harrop, 2013). For this, and as strains tolerant to SO_2 exist (Avramova et al., 2018), alternate antiseptic molecules or methods are looked for by winemakers. The International Organization of Vine and Wine (OIV/OENO 336A/2009, 337A/2009, 338A/2009) and the European Union (Regulation EU 53/2011) authorized the use of fungal chitosan including for organic wine elaboration (Regulation EU 1584/2018, amending the EU 889/2008 Annexes). Chitosan is a ubiquitous polysaccharide composed of β-(1,4)-2-acetamido-2-deoxy-D-glucose repeating units, obtained by chitin deacetylation (Rinaudo, 2006). Fungal chitosan may be considered as a multifaceted aid in winemaking, with antimicrobial, metal chelating, clarifying and antioxidant activities (Brasselet et al., 2019; Castro Marín et al., 2021).

Since its authorization as an antimicrobial agent in wine, many chitosan-based products have been proposed not only to eliminate *B. bruxellensis*, but also other spoilage species or to globally reduce the microbial pressure (Bağder Elmacı et al., 2015; Castro Marín et al., 2020, Marchante et al., 2021; Valera et al., 2017). Some of these products are recommended to reduce the initial microbial population on grapes or must and to modulate the NS yeast consortia. Others can be used after fermentations in order to control LAB or AAB populations. Suppliers recommend these products either as a curative or as preventive agent and racking the wine a few days after treatment (to eliminate both chitosan and the microbial sediment) could be considered as optional, contrarily to the Oenological Codex recommendations (OIV-OENO 368-2009).

Very few recent scientific articles support these practices. Most do not provide any data on fermentation kinetics after early treatment (Castro Marín et al., 2020; Picariello et al., 2020), but Castro-Marín et al. (2018) and Scansani et al. (2020) observed delayed alcoholic fermentations in red wines. In addition, we have recently shown that the intraspecific genetic diversity of *B. bruxellensis* induces variable responses after chitosan treatment (Paulin et al., 2020). Many wine parameters also modulate the effectiveness of the treatment. This may, at least partially, explain why some publications mention the high antiseptic efficiency of chitosan on contaminated wines while others report more modest effects (Bağder Elmacı et al., 2015; Brasselet et al., 2019; Ferreira et al., 2013; Gil et al., 2004; Petrova et al., 2016; Taillandier et al., 2015; Valera et al., 2017).

In this context, our objective was to examine the efficiency of fungal chitosan as an antiseptic on the most representative microbial species present in musts and wines, including fermentative and spoilage species. The studied strains were selected taking into account the genetic diversity of each species. Trials were also conducted at different winemaking stages and on different types of wine, with two different chitosans.

2. Materials and methods

2.1. Microbial strains

A collection of 206 wine microbial strains was studied: 34 *Saccharomyces*, 56 *Brettanomyces*, 29 other NS, 33 *O. oeni*, 43 other LAB, and 11 AAB. Their name, origin and genetic group (when relevant) are indicated in Supplemental data 1.

2.2. Chitosan

Two different fungal chitosan were used, named F1 and F4. Both were sourced by BioLaffort and have been previously characterized (Paulin et al., 2020). Both have a low glucan content (>2 %). F1 has a MW of 32 kDa and a degree of acetylation (DA) of 9.6 %, while F4 displays a MW of 400 kDa and a DA of 15.8 %. F1 perfectly fulfils the oenological codex requirements (Oenological Codex, 2022), while F4 shows a too high viscosity to be a "wine codex accepted" chitosan.

2.3. Culture media and wines used

2.3.1. Solid media for microbial counts during screening on single strains in inoculated wines

YPD solid medium containing 10 g/L yeast extract (Difco Laboratories, Detroit M1), 10 g/L bactopeptone (Difco Laboratories, Detroit M1), 20 g/L D-glucose (Sigma-Aldrich) and 25 g/L agar (Fisher) was used for yeasts plate counts. For bacteria, we used solid grape juice medium containing 250 mL/L commercial red grape juice, 5 g/L yeast extract (Difco Laboratories, Detroit M1), 0.1 % Tween® 80 (Sigma-Aldrich) and 25 g/L agar (Fisher).

2.3.2. Solid media for microbial counts in naturally contaminated wines

For total yeasts (TY) counts, solid medium containing 10 g/L yeast extract (Difco Laboratories, Detroit M1), 10 g/L bactopeptone (Difco Laboratories, Detroit M1), 20 g/L D-glucose (Sigma-Aldrich), 25 g/L agar (Fisher), 100 mg/L chloramphenicol (Sigma-Aldrich) and 150 mg/L biphenyl, pH 4.8, was used. For Non-*Saccharomyces* yeasts (NSY) counts, TY medium supplemented with 500 mg/L of cycloheximide (Sigma-Aldrich) was used.

LAB counts were performed on grape juice solid medium, pH 4.8, completed with 100 mg/L Delvocid (DSM Food Specialities B.V.).

AAB counts were done on grape juice solid medium, pH 4.8, completed with 100 mg/L Delvocid (DSM Food Specialities B.V.) and 2,5 mg/L penicillin (Sigma-Aldrich).

2.3.3. Wines

Several wines and musts were used (Supplemental data 2). When necessary (i.e. if the wine needs to be inoculated with pure strains), the wines were pasteurized for 30 min at 85 °C, before inoculation and chitosan treatment.

2.4. Chitosan treatment

2.4.1. General screening in homemade wine and other inoculated wines or musts

Microbial strains were gradually adapted to pasteurized wines/musts according to Paulin et al. (2020). They were then diluted into wine or must to ensure an initial population comprised between 10^3 and 10^5 CFU/mL. Subsequently, the inoculated wine was distributed into 6 tubes (13 mL/tube). A 100 X aqueous suspension of chitosan F1 was diluted into the two first tubes to reach a final concentration of 40 mg/L (i.e. 4 g/hL). The same was done to the two following tubes using the chitosan F4 and the two remaining tubes were kept as controls. After gentle homogenization, wines were left to settle at 20 °C. After 3 days, 3 tubes (one control and a tube treated with each chitosan) were analysed. The first 12 mL of each tube were gently removed and transferred into a new

tube. They constituted the "racked wines", while the remaining part (1 mL left at the bottom of each tube) was considered as "lees". Both "lees" and "racked wines" were homogenized and analysed for cultivable microbial populations.

After 10 days of incubation at 20 °C, the 3 remaining tubes were analysed the same way.

For each strain examined, the assay was carried out twice, using biological duplicates (different precultures) and two distinct manipulators.

2.4.2. Other tests

2.4.2.1. Tests on grape musts. Each assay was carried out in duplicate on 5 kg of crushed Cabernet-Sauvignon grapes, in small tanks. Controls, carried out in the same must, consisted in sulphiting the must up to 4 g/hL of total SO_2 on one side or no SO_2 addition on the other. After two days at 6 °C (mimicking a traditional pre-fermentation step in red winemaking, PFM), each assay was then inoculated with *S. cerevisiae* strain Zymaflore® FX10 (Laffort) at 20 g/hL (initial population 10^6 ufc/mL). Alcoholic fermentation was monitored every day for 12 days, by density measurement until dryness. Each tank was then racked and inoculated with *O. oeni* strain LACTOENOS® B7 DIRECT (Laffort) at 1 g/hL. Malolactic fermentation was monitored measuring malic acid every week during 1 month, until achievement of malic acid degradation.

Cultivable yeast, AAB and LAB were enumerated at vatting, after pre-fermentary maceration and at the end of alcoholic fermentation.

2.4.2.2. Tests on naturally contaminated wines. The wines were distributed into as many tubes as there were treatments (chitosan F1 at 4 or 10 g/hL, sulphur dioxide at 4 g/hL and untreated control) and samples. The antiseptic molecule studied was then added to the appropriate tube and the tubes were left to settle at 15 °C. A first tube was analysed after racking on day 3. On day 10, all the remaining tubes were racked and the racked wine was transferred to new tubes. One tube of each condition studied was analysed for microbial populations and all the remaining ones were stored at 16 °C to be analysed again after 1, 2 or 3 months. For SO_2 treated wines, the free and total SO_2 were measured in the racked wine according to the oenological Codex, and free SO_2 was adjusted to 27 mg/L before storage, if necessary.

To analyse malolactic fermentation (MLF) feasibility after treatment, on day 10, four tubes of racked wines were withdrawn. Two were inoculated with a lyophilized malolactic starter (LACTOENOS 450 PreAc®, Laffort, for the red wine and LACTOENOS® B7 DIRECT, Laffort, for the white wine), according to the manufacturer instructions, and the two other tubes were left not inoculated. The 4 tubes were incubated at 20 °C. Each duplicate was monitored for malic acid degradation (enzymatic assay, Libios, France) during 40 days.

2.5. Plate growth conditions, cultivable cells count and microbial identifications

Microbial cultivable populations were measured by serial dilutions in physiological serum and plate counts on appropriate solid medium. Three plate counts were done for each sample. For LAB counts, plates were incubated in anaerobic condition with Sachet Oxoid™ (Thermo Scientific™) to inhibit AAB development. All the plates were incubated at 25 °C.

When relevant, microorganisms (30 isolated colonies) were identified at the species level by MALDI-TOF mass spectrometry using *MALDI* Biotyper® (MBT) from Bruker and a homemade database according to Windholtz et al. (2021).

2.6. Reduction factor calculation

From the populations determined by plate counts, we determined the reduction factor (RF), by comparing the population in the racked wine (after 3 or 10 days of treatment) with the initial population in the wine. RF = initial population / population in the racked wine after treatment.

3. Results

3.1. General screening in homemade wine

A total of 206 microbial strains representing 27 species were studied. When the genetic intra-specific diversity had previously been described, strains representative of the species were selected (Supplemental Table 1). The microorganisms were gradually adapted to the homemade wine (HW) until microbial cultivable populations remained stable or increased in the control tubes during the 10 days-experiment (see examples Fig. 1). The initial population was set to about 10^4 CFU/mL. As previously reported (Paulin et al., 2020), after 10 days of experiment, a marked cell enrichment was noticed in the lees fraction compared with the upper compartment (i.e., the racked wine) for most of the yeast species studied, due to natural sedimentation. This phenomenon was much less important in the case of bacteria (Fig. 1 and Supplemental data 3).

Upon treatment with either F1 or F4 chitosan, three distinct behaviours were observed. Examples are presented in Fig. 1 for each microbial group examined. First, there were cases in which the population fell below the detection threshold in both the racked wine and in the lees: this was designed the "sensitive profile". This was observed in all microbial groups examined, except the AAB one. In certain cases, the disappearance of cultivable populations was evident within 3 days, while in other cases, 10 days were necessary. The "intermediate profile" designed trials where cultivable populations remained detectable in the lees although populations in the racked wine fell below the detection threshold. The last profile corresponded to "tolerant" strains. Detectable and often high populations level remained in racked wine after 10 days of treatment with this group. Populations were even higher in the lees. In certain cases, growth was observed between day 3 and 10 and sometimes populations were higher in the treated racked wines than in the control (see examples Fig. 1, in the *Saccharomyces* or in the *Lactobacillus* and *Pediococcus* groups). Indeed, chitosan was inefficient in those cases.

The Oenological Codex recommends racking 7 to 10 days after chitosan addition to efficiently eliminate *B. bruxellensis*. As racking separates lees from wine, strains showing sensitive and intermediate profiles should be eliminated by this procedure. However, for strains displaying a tolerant profile, microorganisms may not be eliminated and persist even in the racked wine. Nevertheless, even within the tolerant profile, significant population reductions could be observed in the racked wine. In this perspective, the treatment efficiency was also evaluated through the overall population reduction factor (RF) after treatment and racking. We stated that a RF greater or equal to 100 indicated an efficient treatment. For each microbial group studied, Fig. 2A presents both the proportion of strains displaying a "sensitive", "intermediate" or "tolerant" profile in the presence of chitosan F1 or F4. It also indicates the proportion of strains displaying a reduction factor higher than 100, hence tempering the tolerant categorization of the considered microorganism.

Whatever the microbial group considered, it appeared that F1 was much more effective than F4. This is particularly obvious with yeast of the *Saccharomyces* genus and for *Pediococci*, for which the proportion of strains displaying a RF > 100 declined from 67 % (F1) to 33 % (F4) and from 53 % (F1) to 0 % (F4) respectively. In addition, considering these two groups, the proportion of tolerant strains increased from 44 % (F1) to 79 % (F4) and from 45 % (F1) to 100 % (F4) respectively (Fig. 2A).

For both chitosan used, the less sensitive microbial group was AAB: only 10 % of the strains studied showed a reduction factor >100 over the 10 days of the experiment and all the strains examined displayed a tolerant profile. Even after racking, the chitosan treatment had a very small impact on AAB populations in the studied wine.

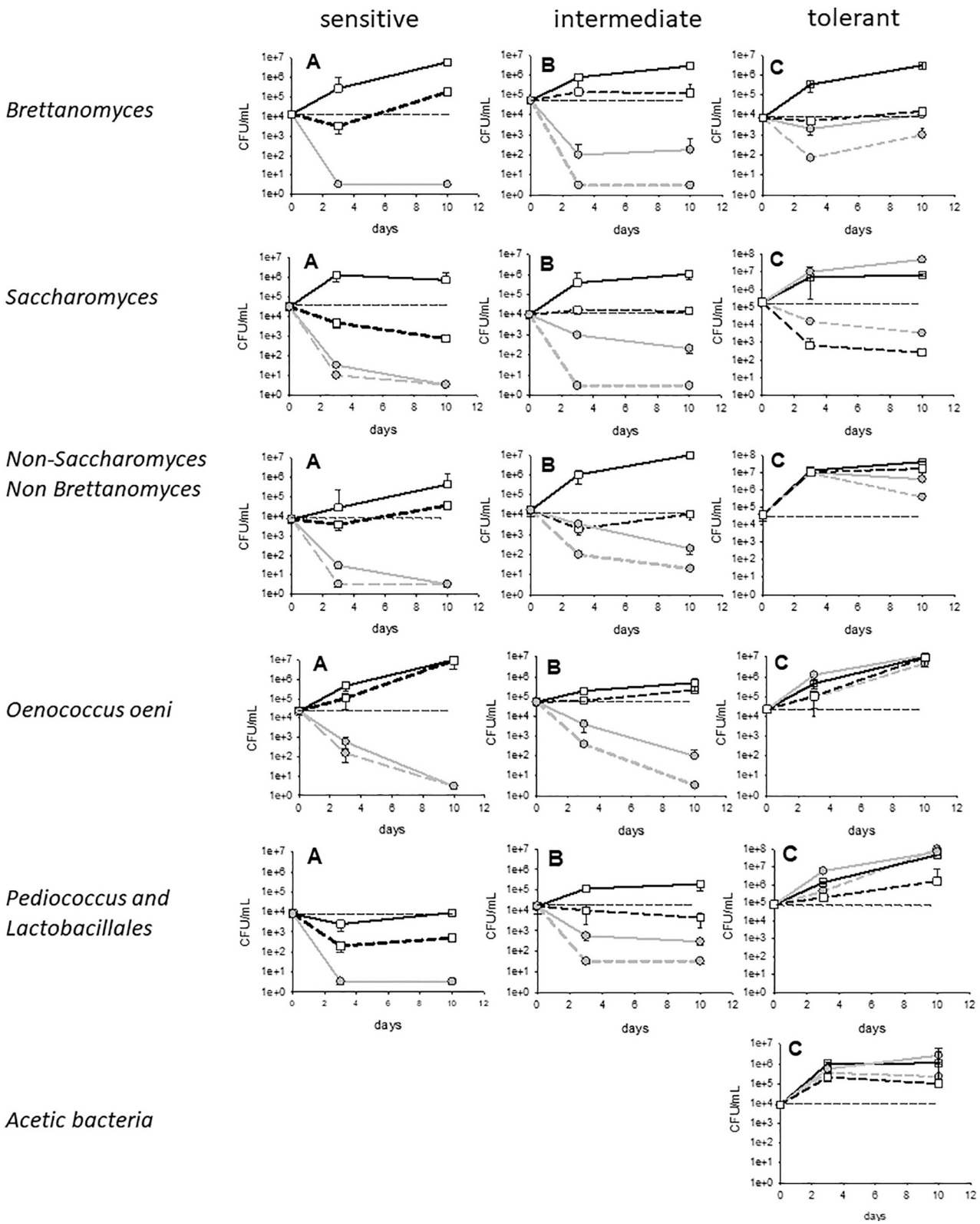

Fig. 1. Examples of sensitive, intermediate and tolerant profiles observed in the presence of chitosan in HW wine. The white squares represent the populations measured in the controls (solid lines: lees; dotted lines: racked wine) and the grey circles represent the populations measured in the assays treated with fungal chitosan (solid lines: lees; dotted lines: racked wine). Each graph presents the results of a single reproducible assay and each point with error bar the mean of the distinct counts obtained after serial dilutions. For each graph, the strain and the chitosan considered are indicated: Group *Brettanomyces*: A. *B. bruxellensis* AWRI1499 (F1); B. *B. bruxellensis* AWRI1677 (F1), C. *B. bruxellensis* AWRI1608 (F1). Group *Saccharomyces*: A. strain Gold (F1), B. Strain L0439 (F1), C. Strain SB (F4); Group *Non saccharomyces*, *non Brettanomyces*: A. *M. pulcherrima* 14106 (F1), B. *Z. bailii* L0446 (F1), C. *H. uvarum* L0764 (F1); Group *Oenonoccus oeni*: A. strain S28 (F1), B. strain IS1491 (F1), C. strain L65.2 (F1); Group *Pediococcus/Lactobacillales*: A. *Secundilactobacillus collinoides* 0203 (F1), B. *Levilactobacillus brevis* ATCC27305 (F1), C. *P. parvulus* 0301 (F1). AAB: C. *Gluconobacter oxidans* 11203 (F1).

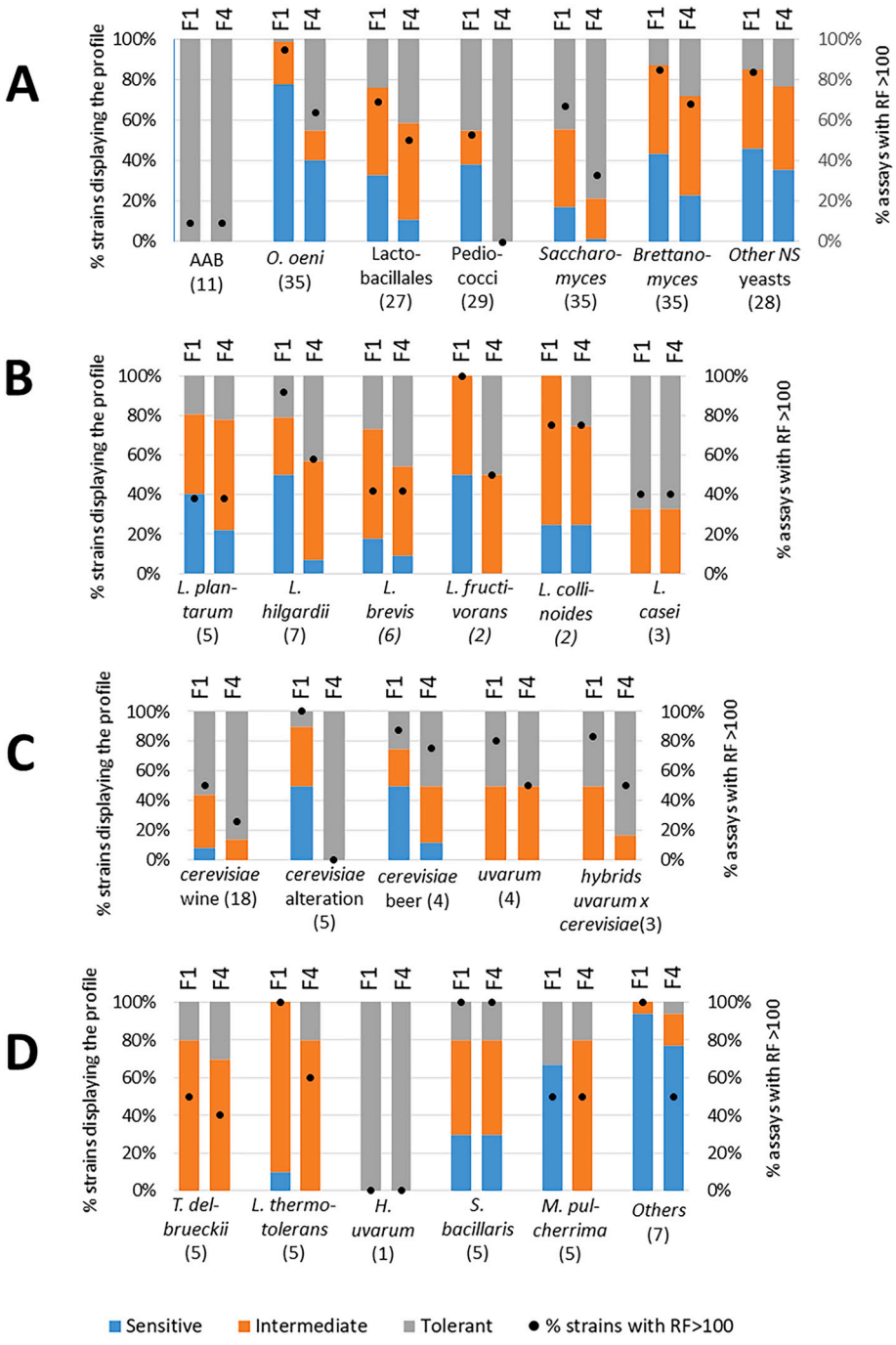

Fig. 2. Proportion of strains displaying a sensitive, intermediate or tolerant profile (cumulative bars) or displaying RF >100 (black dots) in the presence of chitosan F1 or F4 in the HW wine.
A. Comparison of the distinct groups of wine microbe examined.
B. Comparison of species in the *Lactobacillales* group: *L. plantarum* (*Lactiplantibacillus plantarum* subsp. *plantarum*), *L. hilgardii* (*Lentilactobacillus hilgardii*), *L. brevis* (*Levilactobacillus brevis*), *L. fructivorans* (*Fructilactobacillus fructivorans*), *L. collinoides* (*Secundilactobacillus collinoides*), *L. casei* (*Lacticaseibacillus casei*).
C. Comparison of strains and species in the *Saccharomyces* group.
D. Comparison of strains and species in the non-*Saccharomyces* (*NS*) non-*Brettanomyces* microbial group.

On the contrary, the *O. oeni* group gathered predominantly F1 sensitive strains: 95 % of the isolates showed a reduction factor >100 % over 10 days with this chitosan. Chitosan F4 was less efficient on this group as only 64 % of the studied strains showed a RF higher than 100. More than 75 % of the studied *O. oeni* strains displayed a sensitive profile in the presence of F1 and 40 % also presented this type of profile with F4. Most of the *O. oeni* strains studied have previously been shown to produce either capsular or ropy and free exopolysaccharides (Dimopoulou et al., 2014; Dols-Lafargue et al., 2008). No sensitivity significant difference could be demonstrated between strains producing or not exopolysaccharides. Similarly, neither the origin of the strains (red wine, white wine, cider), nor the genetic group seemed to modulate sensitivity to chitosan under these conditions examined (data not shown).

These two groups of bacteria were those within which the behaviours were the most homogeneous. In the *Lactobacillales* and *Pediococci* groups, more contrasting behaviours were noticed. In the *Lactobacillales* group, 69 % of the strains had a RF >100, with F1. Sensitive profiles were observed for 33 % of the strains, 43 % were intermediate and 24 % were tolerant. The *Lacticaseibacillus casei* species seemed to be the least sensitive. No "sensitive" strain was found among the three evaluated, whether with F1 or F4. *Lactiplantibacillus plantarum*, a species sometimes used as malolactic starter, gathers both very sensitive and very tolerant strains (Fig. 2B).

In the *Pediococci* group, 53 % of the strains had a RF >100 in the presence of F1. Among the thirty studied strains, 38 % displayed a sensitive profile in the presence of F1 and 45 % a tolerant behaviour. The proportion of tolerant profiles raised to 100 % in the presence of F4. The ropy *Pediococcus* strains able to produce beta-glucan (Walling et al.,

2005) were not distinguishable from the others in terms of sensitivity/tolerance to chitosan (data not shown).

Among the examined yeast, the *Saccharomyces* group was the least sensitive to chitosan F1, with an overall RF >100 in 67 % of cases compared to 85 % for the *Brettanomyces* group (*B. bruxellensis* and *B. anomalus*) and 84 % for other non-*Saccharomyces* (NS) yeast. The *Saccharomyces* group was also the less affected by the high-viscosity chitosan F4: only 33 % of the strains had an RF >100 in the presence of F4 whereas this percentage rises to 68 % for *Brettanomyces* and 75 % for other NS yeasts (Fig. 2A).

Differences could be noted within the *Saccharomyces* group. The wine fermentation strain subgroup was the most resistant to chitosan treatment: only 50 % of the strains in this subgroup presented a RF >100 in the presence of F1 and 26 % in the presence of F4 (Fig. 2C). The *Saccharomyces* alteration strains subgroup (which gathers film-forming strains or ones isolated from altered sweet wines) systematically showed a RF higher than 100 with chitosan F1. Using F4 chitosan, totally opposite results were obtained: populations levels were even more important in the treated wines than in controls for all strains in the subgroup. All strains in the beer subgroup (beer strains and one isolated from tree exudates) were very sensitive to both chitosan F1 and F4. Finally, the *S. uvarum* strains and the *S. cerevisiae* × *S. uvarum* hybrids showed intermediate RF between wine strains and beer strains, indicating that they are somewhat affected by chitosan. However, their behaviour was frequently tolerant, particularly in the presence of F4.

In the *Brettanomyces* group, *B. anomalus* was clearly more sensitive than *B. bruxellensis*. Indeed, the three *B. anomalus* strains studied showed a sensitive profile with F1 and only one strain showed an intermediate profile with F4 (data not shown). None appeared to be tolerant whereas, under the same conditions 14 % of the *B. bruxellensis* strains were tolerant and 47 % intermediate in the presence of F1. Furthermore 30 % of all *B. bruxellensis* strains examined were tolerant and 50 % showed an intermediate profile in presence of F4.

In the NS yeast group other than *Brettanomyces*, notable differences appeared among species (Fig. 2D). In the *T. delbrueckii* subgroup, 50 % of the studied strains displayed a RF > 100 using F1 and 40 % with F4. Conversely, *Starmerella bacillaris* strains were more sensitive, with strong population reductions in the racked wines: 100 % of the studied strains displayed a RF > 100 in the presence of either F1 or F4. However significant populations could be observed in the lees in 50 % of cases (intermediate profile) and growth occurred during treatment in 20 % of cases (tolerant profile). *Lachancea thermotolerans* appeared to be a sensitive species when considering the proportion of strains showing a RF >100, but similarly to *S. bacillaris*, significant surviving populations were observed in the lees. The other yeast species examined (*Saccharomycodes ludwigii* and species of the *Zygosaccharomyces* genus) were quite well eliminated by a chitosan treatment involving F1, with 94 % of strains presenting a sensitive profile. Finally, *H. uvarum* seems to be absolutely insensitive to any treatment, whether with F1 or F4. In fact, the treated and control population levels were very similar (see example in Fig. 1). However, one should be aware of the low number of strains tested in certain species and notably *H. uvarum*, due to difficulties to obtain sufficiently stable populations in the HW wine.

3.2. Assays in other inoculated wines

Previous studies carried out on *B. bruxellensis* revealed that chitosan treatment efficiency could depend on the type of wine in which it was carried out (Paulin et al., 2020). We therefore conducted additional trials with different wines.

First, the chitosan impact was evaluated in 3 different red wines (HW, AD and D) inoculated with two different *O. oeni* strains (VF and IOEB Sarco-450), using F1 and F4 chitosan. As shown Table 1, strains appeared much less sensitive to the treatment in wines others than the HW one.

Five *S. cerevisiae* belonging to the alteration subgroup (CRBO L0431, L0432, L0433, L0437 and L0439) were inoculated in a white sweet grape must (SW must). This must was treated with chitosan immediately after inoculation or a few days after the beginning of alcoholic fermentation (corresponding to the wine "mutage stage", SW wine). For tests performed in the SW must, racking was not possible, probably due to the intense CO_2 bubble release which prevented sedimentation, and no lethality was observed: all the strains were tolerant. In the sweet SW wine, all tested strains also displayed a tolerant profile, with either F1 or F4 chitosan, although most of the tested strains were sensitive to F1 in the homemade wine (not shown).

3.3. Chitosan treatment for solving oenological issues in naturally contaminated wines

3.3.1. Must microbial control by chitosan as an alternative to SO_2 addition

A rising practice consists in treating not only wines but also grape juice for microbial control, often in a low/no sulphited must context. To gain further insight on this practice effectiveness, we compared the impact of a 4 g/hL F1 chitosan treatment and a SO_2 addition in a red grape must. Microbial populations were examined at vatting (just before SO_2 or chitosan addition), at the end of the pre-fermentary maceration (PFM) and at the end of alcoholic fermentation (Fig. 3).

As shown in Fig. 3A, only the SO_2 treatment was able to lower the yeast populations. Actually, in chitosan treated wines and in the control, total yeast populations increased from 10^5 to 10^6 to nearly 10^7 CFU/mL during the pre-maceration phase. The same result was obtained with NS yeast populations. Likewise, LAB and AAB populations could only be lowered and controlled in the SO_2-treated must (Fig. 3A). Their populations developed in the control and chitosan assays.

No difference in the main yeast species composition could be noticed when comparing these two assays, whereas the sulphited must clearly evolved differently (Fig. 3B). The main difference in between the chitosan/non sulphited assays on one side and the sulphited assay on the other could be ascribed to the higher dominance of *H. uvarum* in the two first modalities. As in the homemade wine, chitosan treatment did not enable to prevent the development of this spoilage yeast. *Acetobacter* was the only genus that could be identified in the non sulphited and chitosan treated must at the end of the PFM step. Populations were too low to conclude as for the genus/species present in the SO_2 treated assay (data not shown). Concerning LAB, species identification did not enable to differentiate the three modalities at the end of the PFM step or at the end of the alcoholic fermentation (data not shown).

Alcoholic and malolactic fermentations kinetics were not affected in neither assay, indicating that treatments did not have any impact on the inoculated *S. cerevisiae* strain, nor on the inoculated bacteria for driving MLF. All together, these results show that chitosan, in our assay conditions, was not an efficient SO_2 alternative for red grape must microbial control in Cabernet-Sauvignon wines.

Table 1
Profiles observed after a F1 or F4 chitosan treatment on 3 different red wines inoculated with two different *O. oeni* strains (initial LAB population: 10^4CFU/mL).

	Chitosan F1 (4 g/hL)			Chitosan F4 (4 g/hL)		
	Wine HW	Wine D	Wine AD	Wine HW	Wine D	Wine AD
VF	Sensitive	Tolerant	Intermediate	Sensitive	Tolerant	Tolerant
IOEB-SARCO-450	Sensitive	Sensitive	Tolerant	Sensitive	Tolerant	Tolerant

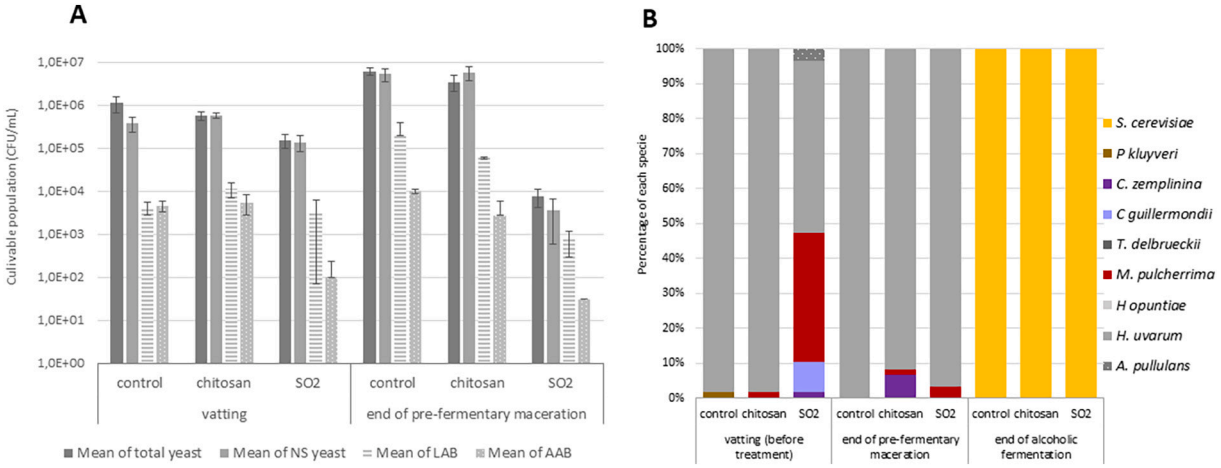

Fig. 3. Comparison of the early treatment of grape must with either chitosan F1 (4g/hL) or SO_2 (4 g/hL) or no antiseptic molecule (control).
A. Evolution of cultivable populations of total and NS yeast, LAB and AAB during the maceration step. B. Inventory of the main yeast species present before PMF, after PMF and at the end of alcoholic fermentation. Results represent the mean of duplicate experiments.

3.3.2. *Wine treatment after alcoholic fermentation*

A red wine (RI) collected just after the end of the alcoholic fermentation and containing an initial NS yeast population of $1.8.10^4$ CFU/mL, an initial LAB load of 6.7. 10^4 CFU/mL and almost no AAB (<10 CFU/mL) was treated with two different doses of chitosan F1. Treated and untreated wines were monitored during 3 months. All microbial populations present in these wines were sensitive to chitosan and the treatment lowered the NS yeast and LAB population below the detection threshold, while significant populations survived in the control wine (Fig. 4A and B).

A few days after racking of both treated and control wines, MLF feasibility was studied. Spontaneous MLF was delayed in the 4 g/hL chitosan treated wine and still not initiated after 40 days in the 10 g/hL chitosan F1 treated wine (Fig. 4C).

MLF begun earlier and took place faster when a malolactic starter was added to the control raked wine or to the 4 g/hL treated wine. But, as for spontaneous MLF, MLF did not begin before 40 days in the 10 g/hLF1 chitosan treated wine (Fig. 4D).

The same experiment was carried out in a white wine (BI) collected at the end of alcoholic fermentation (Supplemental data 4). In this wine

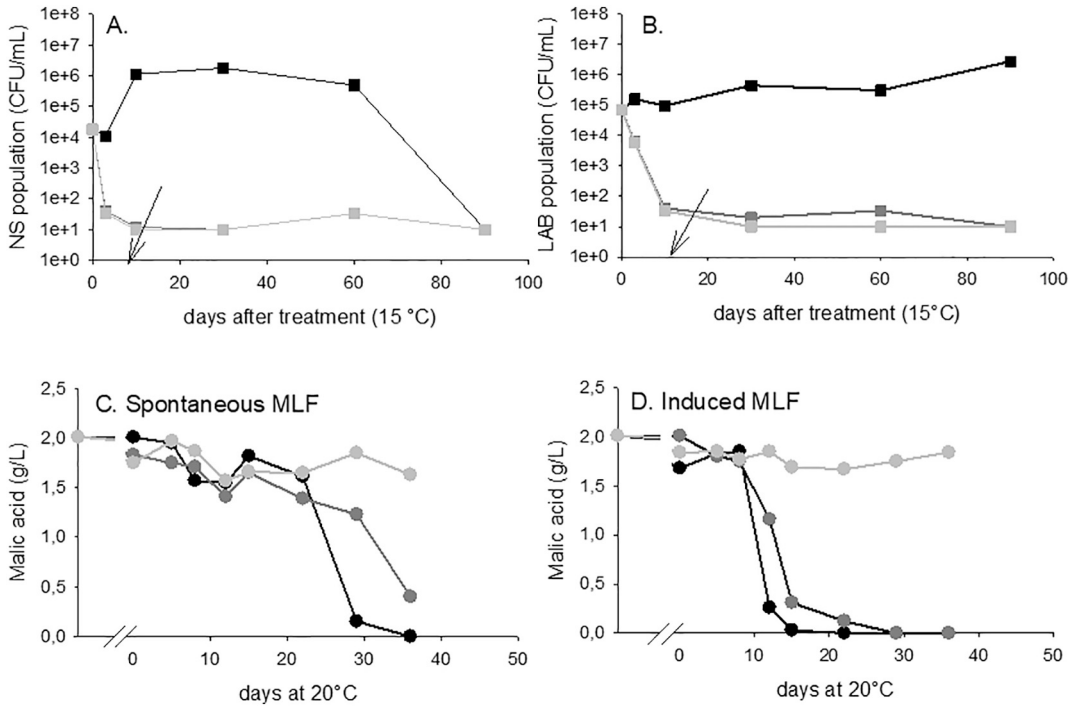

Fig. 4. Effect of chitosan treatment on microbial populations and MLF feasibility in red wine RI. Wine RI was sampled at the end of alcoholic fermentation. It was naturally contaminated by non-*Saccharomyces* yeast and LAB (AAB population <10 CFU/mL). This wine was treated with two doses of Chitosan F1. A and B. After 10 days the wine was racked (arrow) and the racked wine was kept at 15 °C. The populations in the racked wine were followed during 3 months. Black squares: untreated control; dark grey squares: treated with 4 g/hL chitosan; light grey squares: treated with 10 g/hL chitosan. C and D. After racking, samples of the racked treated wines were placed at 20 °C to examine the MLF feasibility during the 50 days following the transfer at 20 °C. We examined either spontaneous MLF with indigenous bacteria or an induced MLF after inoculating the wines with an appropriate malolactic starter on day 0 at 20 °C. Black circles: untreated control wines; dark grey circles: treated with 4 g/hL chitosan; light grey circles: treated with 10 g/hL chitosan.

too, NS yeast and LAB were efficiently eliminated by a chitosan treatment at 15 °C, whatever the dose used (4 or 10 g/hL). Chitosan treatments also delayed MLF whether carried out spontaneously of after malolactic starter inoculation.

In these two wines, the MLF delay even when wines were inoculated with a starter suggests the existence of persistence effects. These could also explain the absence of microbial growth during the three months following the treatment when wines were stored at 15 °C.

3.3.3. *Wine treatment after MLF*

Wine treatment with chitosan was also performed just after MLF in two different red wines, named B and N, naturally contaminated with *B. bruxellensis* (unknown strain) and containing significant LAB populations but almost no AAB (<10 CFU/mL). Chitosan addition was performed at two doses (4 and 10 g/hL) and compared with a SO_2 treatment. Fig. 5 presents the evolution of NS yeast and LAB populations during three months.

In wine N, despite high initial NS yeast and LAB populations (close to 5.10^4 CFU/mL), chitosan treatments appeared very efficient and durable. The NS yeast and LAB cultivable populations remained below the detection threshold over the 3 months following racking (Fig. 5 A and B), whatever the dose of chitosan used. Sulphur dioxide reduced effectively LAB populations but was much less efficient than chitosan for NS yeast population reduction over the long term, probably because of declining active SO_2 over time.

In wine B, the initial NS yeast population was closed to 10^3 CFU/mL. In the control and in the 4 and 10 g/hL chitosan treated wines, these populations progressively grew up to 10^6 CFU/mL. By opposition, the sulphur dioxide treatment (at 4 g/hL) enabled to decrease the NS populations below the detection threshold. However, NS yeast growth was visible during the last month in wine B.

Sulphur dioxide and chitosan at 4 g/hL similarly reduced LAB population, but the most efficient treatment was obtained with 10 g/hL chitosan F1.

To sum up, whatever the chitosan dosage, the treatment had opposite effects on NS yeasts in those wines N and B. The differences between the two wines were less important regarding LAB elimination.

4. Discussion

Chitosan has been initially developed to treat *B. bruxellensis* contaminated wines (International Code of Oenological Practices OIV/OENO, 338A/2009, 2009). This product is now more and more recommended to solve others microbial issues based on the hypothesis that it could also kill or prevent the growth of diverse wine microorganisms.

This study clearly shows that chitosan affects most microbial species potentially found in wine related environments, even when used at low dose (4 g/hL). In the HW wine used for the large microbial collection screening, *O. oeni* and some NS yeast species such as *S. bacillaris* appeared very sensitive, with >90 % of the strains studied displaying a population reduction of more than a 100-fold. By opposition, 90 % of the AAB studied were tolerant to a chitosan treatment. Furthermore, as previously shown for *B. bruxellensis* (Paulin et al., 2020), significant differences in behaviour and susceptibility exist among strains within a same species. Different cellular surface charges could be involved in these variations, favouring or limiting the interactions between chitosan and cells (Taillandier et al., 2015). However, the presence of neutral polysaccharides at the cell surface (beta glucans from ropy LAB such as *P. parvulus* or *O. oeni*) did not significantly change the strain sensitivity, which suggests that they do not mask the potential cell surface chitosan targets.

As previously noted for *B. bruxellensis*, F4 chitosan is much less efficient than the F1 one (Paulin et al., 2020). Its molecular weight may induce a faster sedimentation which probably prevents a proper interaction between chitosan and microorganisms (Strand et al., 2002). The wine itself can also modulate the treatment efficiency: negatively or positively charged wine compounds may behave as competitive inhibitors hence preventing chitosan-cell interactions. Wine composition and parameters like pH, alcohol content can modulate the physiological state of microbes which in turn may affect their sensitivity (Raafat et al.,

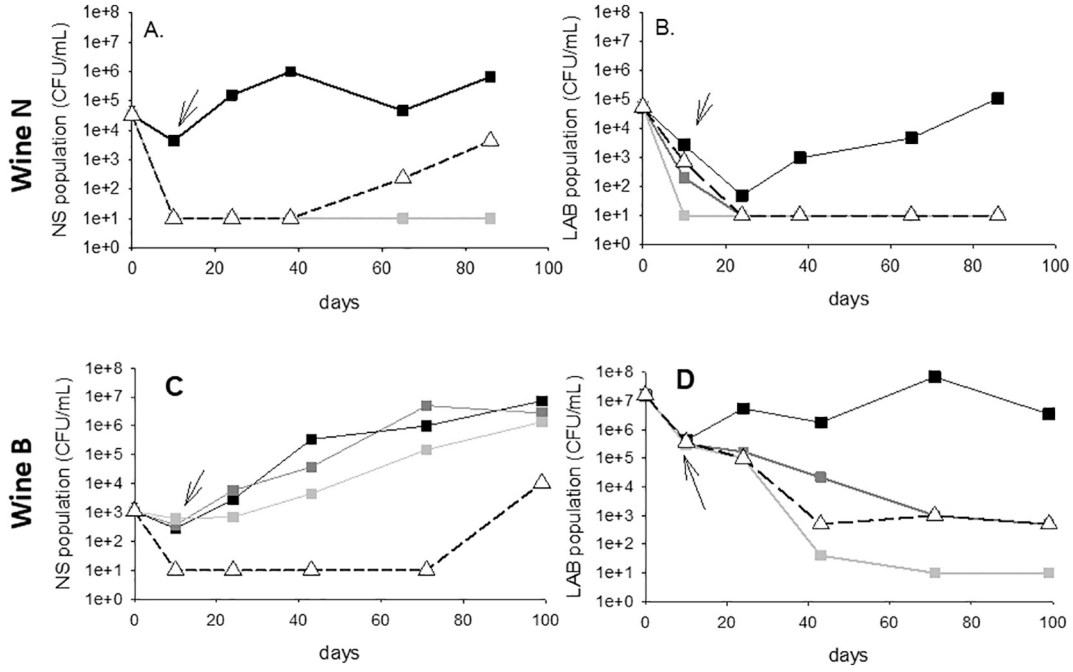

Fig. 5. Effect of a chitosan treatment on microbial populations in red wines after MLF. Wine B and N were naturally contaminated by non-*Saccharomyces* yeast and LAB (AAB population <10 CFU/mL). These wines were treated with two different doses of chitosan F1 or with SO_2. After 10 days, the wine was racked (arrow) and the racked wine was kept at 15 °C. The populations in the racked wine were followed during 3 months. Black squares: untreated control; dark grey squares: treated with 4 g/hL chitosan F1; light grey squares: treated with 10 g/hL chitosan F1; white triangles: treated with SO_2 4 g/hL (and adjustment of the SO_2 dose after racking). A and C: NS yeast populations. B and D. LAB populations.

2008). Between 3.12 and 4.08 (the wine pH extreme values examined here), the wine pH may slightly influence the cell surface molecule ionization (Dimopoulou et al., 2019; Zakrzewska et al., 2007). Furthermore, high sugar concentrations seem to enhance *Saccharomyces* yeast insensitivity to chitosan, probably by modifying yeast metabolism and physiological state. In sweet wines or musts, high viscosity associated with CO_2 release may limit the cell and chitosan sedimentation, thus reducing the treatment efficiency.

As a result, chitosan seems to be adapted to solve some oenological issues related to microorganisms, but not all:

- AAB elimination doesn't seem possible, even in a very permissive wine, like the HW wine used. Outside a wine context, No et al. (2002) suggested that chitosan has stronger bactericidal effects with gram-positive bacteria than gram-negative bacteria.
- Elimination by chitosan of the unwanted *H. uvarum* yeast species, capable of acetic acid and ethyl acetate production and mainly found at pre-fermentation stages, is not possible in the different tests carried out, as already described in Aglianico grape juice (Picariello et al., 2020). Furthermore, treatments at the MPF stage may affect useful species such as *M. pulcherrima*. Additional studies with *H. uvarum* strains tolerant to ethanol would be interesting to definitely state on this point.
- Prevention of sweet wines re-fermentation using chitosan is not possible, nor the mutage of this type of wines. Indeed, in this study, *S. cerevisiae* was never affected by chitosan in a sweet wine context. Indeed, the few S Cerevisiae strains that appeared sensitive were sensitive only in the specific context of the homemade wine.
- Despite a high diversity of LAB responses to chitosan addition in the HW wine, chitosan treatments significantly reduced LAB populations in most of the naturally contaminated wines studied. However, if the aim of chitosan treatment is MLF prevention, chitosan should be used at its maximum dosage. Furthermore, we cannot state about the microbial stability period allowed after the 40 first days following a chitosan treatment.
- *B. bruxellensis* elimination by chitosan before MLF is possible only if the present strains and the wine considered are favourable. This had been previously shown in inoculated wines (Paulin et al., 2020). We confirm this observation with naturally spoiled wines. In this case, the antiseptic effect lasts at least 3 months. Nevertheless, chitosan treatment at maximum dosage at this early winemaking stage can delay or even prevent MLF. If MLF is wanted, it is therefore better to treat wines after, because even a LAB starter addition will not guarantee a MLF success in such early treated wines.
- Finally, *B. bruxellensis* elimination in wines after MLF could also be successful or not depending on the wine considered. Interestingly, in the different wines studied in this paper, treatment of ageing wines containing *B. bruxellensis* significant populations also decreased the LAB populations.

No persistent effects were noticed in wines obtained from treated musts, but the treatment of wines after alcoholic fermentation altered the subsequent LAB growth and MLF onset. This suggests that either the treatment leaves residues in the wine, even after racking, or it eliminates elements essential to microorganism's survival and growth. Indeed, Scansani et al. (2020) observed a slight modification of the final composition of the wines due to chitosan addition. More investigations are needed to precisely know the average preventive period allowed by a chitosan addition, and the microbial spectrum affected by preventive treatments.

5. Conclusion

In some situations, chitosan is completely useless and other antiseptic methods should be privileged. In other cases, chitosan displays long lasting antiseptic effects and is a good alternative to SO_2. It would be interesting to better understand the antiseptic mechanism of chitosan and identify the elements involved in microorganism sensitivity and/or tolerance and wine parameters that modulate chitosan activity. Such investigations would help to set up tools for predicting treatment efficiency.

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CRediT authorship contribution statement

MDL, CD, JM, JC, WA designed the experiment. MP, CMS, LD, JM realized the experiments. CMS, MP, WA, PB, IMP, VM, JC, AVC, JM and MDL wrote and carefully read the paper.

Declaration of competing interest

The authors declare that they have no competing interest.

Data availability

Data will be made available on request.

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