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ORIGINAL PAPER

The controversial relationship between chitosan and the microorganisms involved in the production of fermented beverages

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Abstract
Chitosan is a promising antimicrobial agent available in the beverage industry, because it ensures the control of a wide range of spoilage microorganisms. As chitosan does not alter the characteristics of fermented beverages, it is nowadays widely employed in the wine sector. In this work, an exhaustive chemical characterization of 12 commercial chitosans was performed in accordance with the OIV methods. These analyses made it possible to confirm or determine the animal or fungal origin of the 12 samples. Furthermore, ionic chromatography coupled with an amperometric detector (IC-PAD) confirmed peculiar polysaccharide profiles for fungal and animal-derived chitosans. The antimicrobial activity of chitosans was evaluated against the microorganisms involved in beverage fermentation or capable spoil wine, beer and cider. Chitosans were tested in static and stirred conditions, in a synthetic medium that reproduces fermented beverage conditions, to discriminate against the physical settling of cells and their specific antimicrobial activity. Moreover, the activity of the soluble portion of chitosan was checked by inoculating microorganisms in the media after chitosans removal. The results highlighted the different sensitivity of microorganisms to chitosans, allowing selective control of spoilage agents. However, the yeast and bacteria involved in fermentation were damaged by chitosan, and the synthetic media treated with this molecule showed a less fermentative aptitude. These results suggest that chitosan is a promising tool in fermented beverage production, but an in-depth study of the biochemical interaction between chitosan and food microorganisms is necessary.

Keywords Chitosan · Yeast · Lactic bacteria · *Brettanomyces* · Alcoholic fermentation · Malolactic fermentation

Introduction

In the agribusiness, there are two aspects that consumers particularly appreciate: food healthiness, meaning the absence of exogenous components outside of raw materials, and organoleptic quality. Unfortunately, these two characteristics are in apparent contrast, because large part of the alterations that affect the organoleptic quality and the safety of foods are related to an uncontrolled activity of microorganisms [1, 2], at the same time a large part of the exogenous agents that enter the food productive process are used to control spoilage or pathogenic microbes and to restore the food depreciation due to the preservative treatments.

In the production of fermented beverages, and in particular in cider and wine making, the most commonly employed preservative agent is sulphur dioxide (SO_2) that exerts an antioxidant action and counteracts microbial growth [3]. Sulfur dioxide is a broad-spectrum antimicrobial agent that has an inhibitory effect on a wide range of microorganisms. The level of SO_2 that affects microbes varies according to species, making it possible to counteract the growth of spoilage microorganisms with acceptable levels of interference with the alcoholic or malolactic fermentation [4, 5]. The antimicrobial role of SO_2 is today clearly understood. Only the free forms of SO_2 have a generalized antimicrobial effect, while lactic bacteria are sensitive also to the acetaldehyde-bound form. Sulfur dioxide enters microbial cells and alters the activity of key enzymes, inhibiting cell metabolisms and replication [6–8]. Since only the not dissociated form of sulfur dioxide (H_2SO_3) can freely pass through the cell membrane, its concentration regulates the antimicrobial effect [9]. Consumers demand products with a low content of SO_2, due

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to its undesirable effects on human health, therefore SO_2 concentration in fermented beverages is frequently too low to counteract spoilage microorganisms. In oenology, the loss of efficacy of SO_2 is also related to the environmental changes. Global warming causes loss of grape acidity and wines having frequently pH higher than 3.50, with a reduction of the H_2SO_3 portion, that makes sulphur dioxide ineffective against wine spoilage microorganisms such as *Brettanomyces bruxellensis* and wild bacteria [10].

For these reasons, food technologists are looking for new instruments against spoilage microorganisms. The exploitation in winemaking and, more generally, the application in the fermented beverages industry, of the antimicrobial action of chitosans raised a lot of interest [11]. Chitosan is a polycationic linear polysaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine, employed in different industrial fields such as human nutrition [12], as antibacterial agent and coadjutant for drug delivery [13]. In agriculture, its antimicrobial action was exploited in bio pesticides [14], entering also in composite coatings for a wide range of applications in food, feed and water treatment [15]. In winemaking, chitosan has long been used as an antioxidant, in the clarification, fining and chelation of heavy metals [16, 17]. More recently, empirical observations highlighted its capacity to counteract the main spoilage agent of wine, *B. bruxellensis* [18]. The application of chitosan for microbiological control purposes during the production of fermented beverages is particularly interesting, because its efficiency is independent from the chemical features of the beverage, and, in particular, from the pH in the characteristic interval of wine, beer or cider. In addition, chitosan is removed after treatment, apparently not residing in the finished foods [11, 14, 18].

A confirmation of the interest of the agri-food industry towards chitosans is given by the market projections on this class of molecules. [19] The global chitosan market size was valued at $ 1.7 billion in 2019, and is projected to reach $ 4.7 billion by 2027, growing of the 14.5% from 2020 to 2027. Deepening the analysis of the commercial trend of chitosans, the use in the beverage industry is the 3rd most important sector, after water treatment and pharmaceuticals /cosmetics. The beverages segment is expected to witness steady growth due to growing demand for chitosan to improve the quality and shelf life of these products.

Regarding the antimicrobial activity of chitosan, a large bibliography about pathogenic bacteria and molds is available. In synthesis, at pH below 7.00, a large part of the amino groups in the C-2 position of the glucosamine units will be protonated. This cationic charge density triggers the reaction of chitosan with components of the cell wall. In the case of Gram negative bacteria, the target is the anionic surface of the lipopolysaccharide leaflet of [19], while in Gram positive bacteria a reaction with anionic peptidoglycans is observed. The degree of acetylation of chitosan and the pH of the medium determines the charge density [20] and thereby the level of antibacterial activity. Lowering the pH below 5.00, the usual interval of main fermented beverages, increases the antimicrobial effect of chitosans due to a higher proportion of charged amino groups [21–23]. Furthermore, the ionic state of solution determines the strength and range of the electrostatic interaction between the positively charged chitosan and the negatively charged bacteria. Another variable that may influence the degree of interaction is the molecular weight (Mw) of chitosan [24]. Chitosan exhibits also antifungal activity, exploited in the protection of crops and in the contrast of food spoilage yeasts. Different authors observed that chitosan increases cell membrane permeability, through the interactions of its positive amino groups with the negative charges on the phospholipid bilayer of microorganisms [25, 26]. Marquez et al. [27] deepened the knowledge about the mechanism of chitosan action, by demonstrating the capacity of this molecule to interfere with the protein synthesis.

The chitosan employed in the industry derives principally from crustaceans; however, some issues about its potential allergenic effect do not recommend its use in the food sector [28]. Considering the large employment of chitosan in winemaking, the International Organization of Vine and Wine (OIV) has allowed the use of chitosan from fungi in winemaking, and has issued specific methods for discriminating between chitosans from marine or fungi sources. Residual glucan content, viscosity of chitosan in 1% solution and settled density (following settlement) are indicated as discriminant parameters [29]. Chitosan is added to the wine to control *B. bruxellensis* during aging, and removed by raking after 7–10 days to exploit, in addition to the cytotoxic action, their ability to separate cells from wine by sedimentation [18]. However, interest in a wider use of this molecule is growing. The use of chitosan is proposed in the control of bacteria in the early stage of winemaking, its addition was tried during alcoholic fermentation or on grapes, before the harvest [30]. The results is that chitosan residues thought the winemaking and may interact with microbes that cooperate in wine production [31].

Knowledge of this aspect is still modest and often discordant. Further study is necessary, because it is conceivable that chitosan interferes with useful microorganisms such as *Saccharomyces cerevisiae*, other fermentative yeasts or lactic bacteria (LAB) responsible for malolactic fermentation. In this work, the authors wanted to test the effect of different chitosans now available on the market on a wide range of microorganisms related to the production of fermented beverages, both because they are essential for wine fermentations, and because they are potential spoilage agents. The results obtained, thanks to the use of advanced analytical techniques, both in the microbiological and chemical fields,

shed light on the complex interactions between this molecule and microorganisms that turn out to be useful not only in winemaking but to the whole community of fermented beverage producers.

Materials and methods

Chitosan samples, characterization and chemical analysis

In this study, 12 chitosans from different sources were considered (Table 1): 6 from fungi and 6 from animal exoskeletons were provided by different, international suppliers. The declared purity of the samples was over 95% excluding two samples containing citric acid (chitosan purity 60%) and bentonite (chitosan purity 25%).

The fungal or animal origin of the 12 chitosan samples was confirmed according to the official International Organization of Vine and Wine (OIV) standards determining residual glucans, viscosity, tapped density and solubility [29]. The residual glucan content in chitosan was measured spectrophotometrically (UV–Visible spectrophotometer, Cary 100, Varian, US). The procedure is based on a colorimetric reaction in which the response is dependent on the degradation of glucans with concentrated sulfuric acid. This degradation gives rise to a yellow-brownish compound, whose intensity is proportional to that of the residual glucan. The calibration curve was prepared starting from a 5000 mg/L beta-glucan (purity ≥ 95%; Sigma Aldrich, St. Louis, MO) water/ethanol solution (92.5:7.5, *v/v*). This solution was first diluted 25 times with 1% acetic acid (acetic acid:water, 1:99, *v/v*) and secondly, 7 beta-glucan concentration points (0–10–30–50–70–140–175 mg/L) were prepared by diluting the latter solution appropriately with water. The samples were prepared by weighing 100 mg of chitosan into a 50 mL flask and adding 25 mL of 1% acetic acid solution. They were left to stir for 12 h (Multi Reax, Heidolph Instruments GmbH & Co. KG, Schwabach, D), brought to volume with 1% acetic acid and centrifuged (10 min at 4100 rpm; IEC CL31 Multispeed, Thermo Scientific, Sunnyvale, CA) to eliminate any suspended solid residues. Finally, 1 mL of each standard beta-glucan calibration point and each chitosan sample was added to 1 mL of 5% phenol (> 99%, Sigma Aldrich) solution and 5 mL of concentrated sulfuric acid (98%, Sigma Aldrich),they were then stirred for 10 s and left to cool for an hour. The solutions obtained were placed in disposable plastic cuvettes and analyzed with a spectrophotometer at 490 nm against water. The results were expressed in $\mu g_{glucans} \times g_{chitosan}^{-1}$.

The viscosity of chitosan was determined with a rotational viscometer (Fungilab PRO SERIES, Geass, Torino, I). Samples (0.25 g) were weighed into a 25 mL flask, dissolved in 1% acetic acid and centrifuged to remove any suspended solid residues. The thermostated sample (20 °C, 18 mL) was introduced into the viscometer using an adapter for low viscosity reading (LCP Fungilab, Geass). The results were expressed in cP.

Tapped density was measured according to the International Pharmacopoeia method (World Health Organisation, Document QAS/11.450, 2012). Chitosan (10 g) was placed in a 20 mL volume cylinder hooked onto a mechanical tap density tester, setting the engine speed to 250 rpm. Volume readings were taken until little further volume change was observed (the difference between 2 consecutive readings had to be lower than 2%). Tapped density was calculated by dividing the weighted mass (expressed in g) by the final powder volume (measured in mL).

Table 1 Chemical characteristics of chitosans products involved in the experiments

Chitosan	Residual of glucans (%)	Viscosity of chitosan (cP)	Settled density (g/mL)	Detected origin	DD (%)	DA (%)	Mw (KDa)	Solubility* (%)
1	8.4	3.39	0.9	Fungi	74.3	25.7	157	< 5
2	2.2	2.87	0.3	Animal	75.2	24.8	20.5	< 5
3	8.8	3.25	0.9	Fungi	74.0	26.0	277	< 5
4	0.5	65.1	0.5	Animal	68.5	31.5	3910	< 5
5	0.4	79.0	0.4	Animal	76.8	23.2	949	< 5
6	8.6	3.37	0.9	Fungi	75.4	24.6	250	< 5
7	1.9	3.82	0.3	Animal	75.8	24.2	35.5	< 5
8	0.4	52.4	0.4	Animal	78.0	22.0	730	< 5
9	7.9	2.65	0.8	Fungi	71.1	28.9	365	< 5
10	0.4	22.5	0.4	Animal	81.7	18.3	398	< 5
11	13	1.64	1.0	Fungi	72.0	28.0	69.3	< 5
12	11	3.20	0.9	Fungi	75.8	24.2	19.6	< 5

DD deacetylation degree; *DA* acetylation degree; *Mw* molecular weight; (*) = calculated on the pure chitosan content

Solubility of chitosans was checked by dissolving 5 g (dry weight) in 100 mL of ultrapure water and shaking for 2 min. Afterwards, the samples were centrifuged (10 min at 4100 rpm) and the supernatant removed. To eliminate the residual liquid, they were placed in a vacuum oven at 40 °C overnight. The final weight had to be more than 95% of the initial weight (solubility no higher than 5%; OIV [35]). The soluble fraction, dried in in a vacuum oven at 40 °C overnight was then re-dissolved in water:methanol (80:20, *v*/*v*) and analyzed using an ionic chromatograph ICS 5000 (Dionex-Thermo Scientific, Waltham, MS) coupled with an amperometric pulse detector (PAD) with a gold working electrode and a palladium counter electrode. The separation was performed on a CarboPac PA200 analytical column (3 × 250 mm; Dionex-Thermo Scientific). Isocratic separation was achieved with a KOH 0.5 N and flow rate was set at 0.4 mL/min [32]. Solubility was also checked in a water:ethanol (88:12, *v*/*v*) solution at pH 0.350, adjusted using tartaric acid (Sigma Aldrich) to reproduce the conditions usually observed in fermented beverages, wine in particular.

The acetylation degree (DA) of chitosans is obtained calculating the ratio of the number of N-acetyl-glucosamine units to the number of total monomers. It is determined by potentiometric titration of the amino groups following the method described by Rinaudo et al. [33]. About 0.1 g (Md) of dry weight chitosan sample were dissolved in 3 mL of 0.3 M HCl and 40 mL of water and stirred for 12 h, inducing the positive charge of the amino groups. The solution was titrated by NaOH 0.1 M to reach pH 4.50 (V1) corresponding to pKa -2 of the fraction of free amines. The titration was continued until a pH of 8.50 was obtained (V2), corresponding to pKa + 2.

$$DA = \frac{(1 - 162 \times Q)}{(1 + 43 \times Q)}$$

When Q is the number of moles of glucosamine part groups referred to weight unit (g).

$$Q = \frac{(V2 - V1) \times 0.1}{1000 \times Md}$$

Molecular weight was determined by adapting a literature method [34]. Four concentrations of 1.7, 3.3, 6.7, and 10 mg/L chitosan solutions were prepared. Differently from the Chen and Hwa [34] determination, that measured the intrinsic viscosity through a capillary viscometer, our study was conducted by evaluating the kinematic viscosity, obtained with the help of a rotational viscometer. The intrinsic viscosity assumes the value of the intercept for C=0 in the Huggins equation (HE).

$$HE = \frac{\eta_{sp}}{C} = [\eta] + k'[\eta]^2 C$$

where k' is the Huggins constant, C the chitosan concentration (g/mL) and η_{sp} is the specific viscosity of the solution. Specific viscosity is defined as the product between the inverse of the viscosity of the solvent and the difference in the viscosity of the chitosan (η) solution of our chitosan with the viscosity of the solvent (η_0).

$$\eta_{sp} = \frac{(\eta - \eta_0)}{\eta_0}$$

Malic acid in juice and wine was analyzed according to the OIV methods [35] using a Miura enzymatic analyzer (Exacta Optech, Modena, Italy).

Microorganisms and microbiological analysis

The microorganisms involved in the experiments belonged to the German Collection of Culture and Microorganisms (DSMZ) and to the ARS Culture Collection (Table 2). In addition, yeasts isolated in Italian's wineries by the Edmund Mach Foundation (FEM) were employed, identified by sequencing the D1/D2 domain of the large subunit 26S rRNA [36]. The sequences (600-pb) were compared with those available in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/). According to the requirements of the different species, microorganisms were cultured in Wallerstein

Table 2 Intensity and variability of the ion chromatographic peaks that were found peculiar to fungal chitosan

	Retention time (min)	Fungal chitosan (*N.* = 6)			Animal chitosan (*N.* = 6)		
		Lower (nC*min)	Median (nC*min)	Higher (nC*min)	Lower (nC*min)	Median (nC*min)	Higher (nC*min)
Peak 1	7.4	0.069	0.190	0.260	< 0.010	< 0.010	0.020
Peak 2	8.0	0.090	0.178	0.670	< 0.010	< 0.010	< 0.010
Peak 3	12.4	0.083	0.142	0.267	< 0.010	< 0.010	0.048
Peak 4	13.4	0.136	0.247	0.700	< 0.010	< 0.010	< 0.010

Laboratory Medium (WL, Oxoid, Waltham, MA) or in the De Man Rogosa Sharpe Medium (MRS, Oxoid) at 25 °C. ADY were collected on the Italian's market in the harvest 2018–19, stored as listed by the producers (Lallemand, Montreal, CA; Ever, Castel d'Azzano, I; Anchor Yeast, Johannesburg, ZA; La Food, Fasano, I) and reactivated following the OIV standard [38].

Flow cytometric analysis of yeasts

The concentration and viability of the yeast cultures were determined by flow cytometry [37]. 1 mL of sample containing no more than 10^5 of cells, obtained by an appropriate dilution in PBS buffer, was incubated for 10 min at 20 °C in presence of 10 µL of fluorescein diacetate solution (FDA, Sysmex, Kobe, JPN). After incubation, 10 µL of propidium iodide solution (PI, Sysmex) were added to the sample. The FCM analysis was performed using a CUBE 6 Cytometer (Sysmex) equipped with a solid blue laser (488 nm). Thanks to four band-pass filters, four signals were considered: forward-angle light scatter (FSC), side-angle light scatter (SSC), green fluorescence signal (530 nm, FL1 channel) and red fluorescence signal (630 nm, FL2 channel). The FCM analysis was performed using logarithmic gains and specific detector settings, adjusted on a sample of unstained *S. cerevisiae* ATTC 9763 to eliminate the background and the cellular auto fluorescence. Data were collected and analyzed using the FCS Express 4 software (De Novo Software inc., Pasadena, CA). The yeast cell population was identified and gated in the dot plot FSC/SSC; live and dead cells differentiation was performed in the dot plot FL1/FL2 adjusted by the appropriate compensation between the two signals considering the subpopulation of yeast gated in the dot plot FSC/SSC.

Bacteria analysis by plate count

Lactic acid bacteria (LAB) concentration was determined by plate count according to the [38]. The analyses were performed using the De Man Rogosa Sharpe Agar Medium (MRS agar, Oxoid) as synthetic media for LAB growth, incubated at 25 °C under anaerobic conditions (Anaerogen Kit, Oxoid).

Test about the effect of chitosan against microorganisms

Tests of the sensitivity of microorganisms to different chitosans were performed in YM broth medium (Yeast extract 3 g/L, Malt extract 3 g/L, Glucose 7 g/L, Peptone 5 g/L, Oxoid), adjusted with 10% *v/v* of ethanol (Sigma Aldrich), having pH-regulated at 3.50 using L-malic acid (Sigma Aldrich). The addition of ethanol and the adjustment of pH, although not toxic to microorganisms, are necessary to regulate the solubility of chitosans as in fermented beverages. The tests were performed in sterile glass bottles containing 250 mL of YM broth. Chitosans were singularly added 24 h before the inoculum of microorganism at a concentration of 0.5 g/L, the usual amount utilized in oenology [11]. Microorganism was inoculated in the medium at different concentrations, between 2 and the 8 log units/mL, following the experimental plan discussed in the next chapter. In dynamic tests the solution was maintained under continuous agitation by a RH basic magnetic stirrer (IKA Werke, StaufenimBreisgau, D) at 200 rpm. The static test was performed in the same conditions, but without agitation. All experiments were incubated at 25 °C for 3 or 7 days, according to the different growth ratio of microorganisms as indicated by the OIV [35] standard.

Experiments to verify the effect of soluble portion of chitosans were performed adding chitosan to the medium in a concentration of 0.5 g/L and maintaining the solution for 24 h at 3 °C under continuous stirring (250 rpm). After contact chitosans were removed by centrifugation of YM broth at 4000 rpm for 10 min (5 °C) using an Eppendorf 5804R centrifuge (Eppendorf, Hamburg, D). The clear medium was poured into a sterile glass bottle and inoculated with microorganisms after 24 h, as described in the experimental plan (Table 5).

The alcoholic fermentation (AF) tests were performed using 3 different strains of yeast (*S. cerevisiae* ATCC 9763, *S. bayanus* DSMZ 70547 and *T. delbrueckii* DSMZ 70526) inoculated (0.01%, final concentration 10^5 cell/mL) in the YM medium adjusted to increase the glucose concentration to 20% (W/W) and to regulate pH at 3.50. The experiments were performed in sterile glass bottles (1 L of volume), filled with 750 mL of medium and closed with a bubbler; chitosans were added to the medium 24 h before inoculum of yeast. The kinetic of fermentation was followed by a daily measure of the weight loss due to CO_2 evolution using an 4202-1S balance (Sartorius Stedim, Göttingen, D). Malolactic fermentation (MLF) tests were performed in MRS broth (Oxoid) adjusted with 12 g/L of L-malic acid (Sigma Aldrich) and pH 3.50. The treatment of chitosan and inoculum of microorganisms were performed at the same ratio of AF tests using *O. oeni* ATCC 27311, *L. plantarum* NRRL B-1927 and *P. damnosus* LMG 28,219. The determination of L-malic acid was performed by enzymatic assay as described in paragraph 2.1.5.

Statistical analysis

Statistical analysis was carried out using Statistica 7.1 software (Stat Soft Inc., CA). One way ANOVA and Tukey tests were used to identify significant differences between the results of oenological tests.

Results

Chemical characterization of chitosan

Table 1 reports the chemical characteristics of the chitosan products. According to the OIV prescriptions chitosans n° 1, 3, 6, 9, 11, and 12 were confirmed as being of fungal origin, while n° 2, 4, 5, 7, 8 and 10 as coming from animal sources. The DD % ranged between 68.5 (chitosan n° 4) and 81.7 (n° 10) whilst the Mw ranged between 19.6 (n° 12) and 3910 (n° 4). Unfortunately, these two parameters did not seem correlate with the origin of the chitosan samples (R^2 0.344 and p-value 0.258 for correlation with DD and R^2 0.394 and p-value 0.119 for Mw; Two-tailed biserial correlation).

As regards the solubility of chitosans, test performed according to OIV standards (OIV, 2019) confirmed a below 5% solubility for all samples. The test of solubility performed in an ethanol–water acid solution, which creates conditions similar to those observed in fermented beverages, does not deviate from these results. In these conditions, the solubility of the samples varied from 1.2% of sample 4 to 1.9% of sample 11.

Ion chromatographic separation confirmed interesting differences in the polysaccharide profiles of the two categories of chitosan (Fig. 1). The fungal origin was significantly characterized by 4 peculiar peaks at 7.4, 8.0, 12.4 and 13.4 min, which were not detectable or negligible in animal samples. Table 2 reports the peak intensity and variability measured for the two groups.

Sensitivity of microorganism to chitosan.

Table 3 lists the results of the test of sensitivity of 13 microorganisms involved in the fermented beverages production to a commercial formulate of chitosan of fungal origin largely employed in the beverage industry. The initial cell concentration was adjusted at 7.0 ± 0.2 log cell/mL by an appropriate dilution of pure cultures of microorganisms. In the dynamic test, we observed a substantial reduction of the *S. bayanus, L. plantarum* and *P. anomala* population, whereas *B. bruxellensis, S. pombe, C. stellata* and *S. ludwigii* were not detectable (Table 3, detection limit 2.3 log cell/mL). For the other microorganisms tested, in particular *S. cerevisiae* and *O. oeni*, the results are controversial because the microbial population observed at the end of tests oscillated around the initial value, indicating a poor influence of chitosans on cell viability. In the static test, the absence of agitation causes the settling of chitosans a few hours after the addition. The absence of a prolonged dispersion of chitosans resulted in a different behavior of microorganisms. All species were detectable and the decrease in cell concentration, compared to the initial load, did not exceed the 2 logarithmic units (Table 3). *S. cerevisiae* and *T. delbrueckii* showed a noticeable growth with respect to the initial concentration, reaching the 8 log units/mL after 3 days of incubation; other yeasts also affected by chitosans in the dynamic test showed a high cell density in the static test, in particular *S. bayanus, S. pombe, P. anomala* and *S. ludwigii*. Bacteria did not appear to be influenced by the physical state of the medium; their concentration in the two tests was quite similar. Comparing the entire set of data of the two experiments, the constant stirring of the medium resulted in an average concentration of the microbial population of 6.89 ± 7.14 log cell/mL (68% of initial concentration), with a large variability among species (RSD 177%).The static test showed a mean concentration of 7.58 ± 7.74 log cell/mL (4 times the initial concentration) with a moderate variability (RSD 136%). The discrimination between dynamic and static test resulted statistically significant at the ANOVA one way test ($F_{1,24}$ 4.259, p: 0.042).

Considering the relevance of *S. cerevisiae* in the production of fermented beverages, the effect of chitosans on this species was increased, taking into account 16 *S. cerevisiae* strains in the form of Active Dry Yeast (ADY) commonly used in the industry of fermented beverages. Figure 2 shows the amount of viable/dead cells of yeast counted in modified YM broth 24 h after chitosans addition. The initial yeast cell

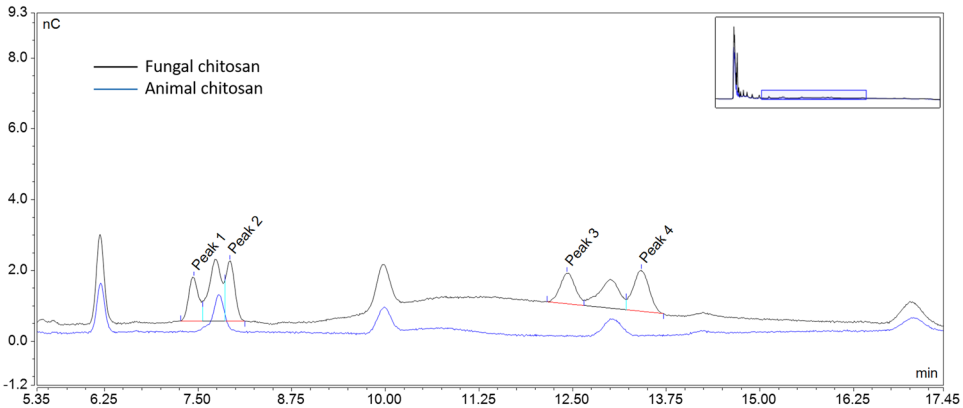

Fig. 1 Comparison of soluble polysaccharide fraction of fungal and animal chitosans analyzed with IC-PAD

Table 3 Effect of the addition of a commercial chitosan of fungal origin against microbes having oenological interest

Specie	Strain	Dynamic test	Static test
		Log cell (UFC[c])/mL	
Saccharomyces cerevisiae[a]	ATCC 9763	7.42 ± 6.45	8.2 ± 7.42
Saccharomyces bayanus[a]	DSMZ 70547	2.69 ± 1.48	7.5 ± 6.73
Brettanomyces bruxellensis[b]	ATCC 52304	nd	5.6 ± 4.86
Torulaspora delbrueckii[a]	DSMZ 70526	7.64 ± 6.78	8.1 ± 7.57
Oenococcus oeni[b]	ATCC 27311	6.82 ± 5.42	5.0 ± 5.31
Pediococcus damnosus[a]	LMG 28219	6.64 ± 6.02	5.2 ± 4.82
Lactobacillus plantarum[a]	NRRL B-1927	3.55 ± 3.42	3.5 ± 2.39
Acetobacter aceti[b]	ATCC 15973	6.74 ± 6.19	6.1 ± 5.10
Gluconobacter oxidans[b]	NRRL B-72	6.82 ± 6.32	6.5 ± 5.00
Schizosaccharomyces pombe[a]	ATTC 24843	nd	7.7 ± 6.68
Candida stellata[a]	FEM	nd	6.5 ± 6.72
Pichia anomala[a]	FEM	1.98 ± 2.20	8.1 ± 7.51
Saccharomycodes ludwigii[a]	FEM	nd	7.6 ± 7.74
Mean ± sd		6.89 ± 7.14	7.58 ± 7.74
Rsd		177%	136%

Test conducted in YM medium using pure cultures of each microorganism having a nominal concentration of 7 log cell/mL for both microorganisms (mean ± SD $n = 3$)

Nd. not detectable (< 2 log units)

Incubation time:[a]3 days; [b]7 days

[c]Bacteria were enumerated by plate count

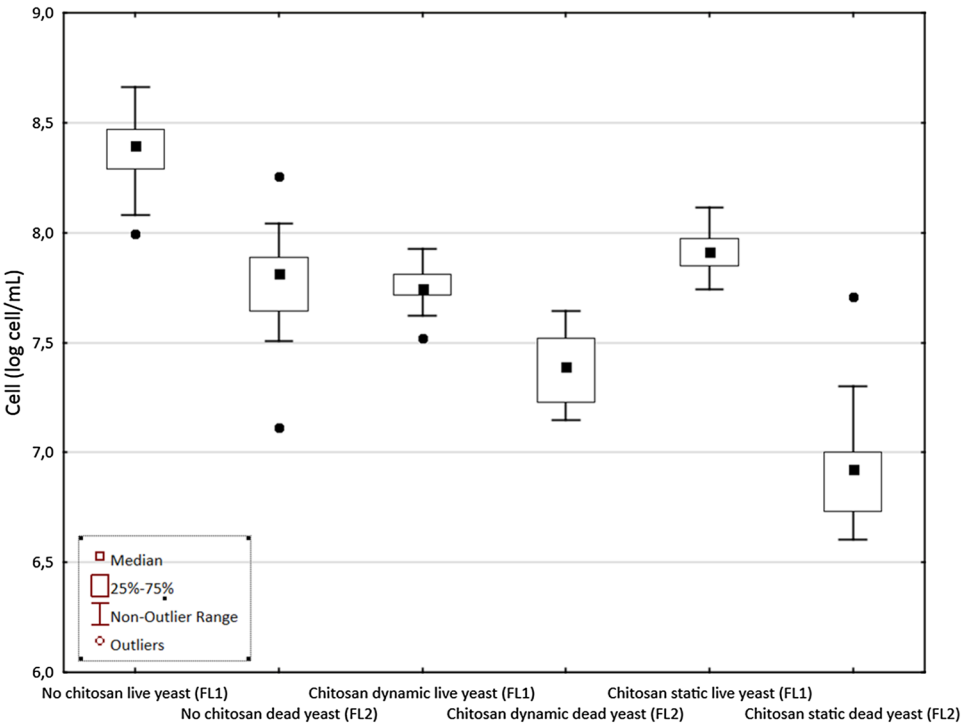

Fig. 2 Box plot of the yeast concentration measured by flow cytometry of different ADY purchased on the Italian's market (n = 16) inoculated in YM (pH 3.50. 7% of glucose) supplemented by a commercial chitosan of fungal origin. Measures of cell concentration were performed before and 24 h after the chitosan addition

concentration was set at 8.00 log cell/mL by appropriate decimal dilution, considering the OIV requirements in terms of ADY viable yeast cell [38]. In absence of chitosans live cells, concentration was 8.38 ± 0.18 log cell/mL on average, and dead cells reached 7.76 ± 0.26 log cell/mL. The addition of chitosans caused a reduction of the concentration of live cells by 0.6 log unit for the dynamic test (7.75 ± 0.10 log cell/mL) and 0.4 log unit in the static test (7.90 ± 0.10

log cell/mL). Dead cells increased in the dynamic test up to 7.98 ± 0.16 log cell/mL, while in the static test a decrease of dead cell population (6.93 ± 0.29 log cell/mL), attributable to the settling due to chitosan was observed. The full factorial 2-way ANOVA test confirmed the significance of the chitosan treatment against *S. cerevisiae* both for live ($F_{2,90}$: 3.097, p: 1.886 E-23) and dead cells counts ($F_{1,90}$: 3.947, p: 1.392 E-28) and also regarding the differences observed in yeast counts performed onto stirred and static experiments ($F_{2,90}$: 3.097 p: 1.151 E-07).

Fermentation test in presence of chitosans

The AF performance in presence of chitosans was evaluated considering three yeast strains belonging to the *S. cerevisiae, S. bayanus and T. delbrueckii* species, usually employed in the industry of fermented beverages (Fig. 3A). The addition of chitosans delayed the lag phase of *S. cerevisiae* of 24 h as against the experiment without them (45 *vs.* 21 h). The maximum alcoholic fermentation rate (V_{max}) was also affected by the addition of chitosans. In the absence of these molecules, V_{max} reached 0.92 ± 0.12 g/L h^{-1} on the 3rd day of fermentation *vs.* 0.88 ± 0.09 g/L h^{-1} (4th day of AF) observed in the experiments treated with chitosans. The addition of chitosans to AF in the form of *S. bayanus* caused a delay of 2 days in the start of AF and a V_{max} of 0.42 ± 0.09 g/L h^{-1} (10th day of AF) as against a V_{max} of 0.84 ± 0.14 g/L h^{-1} reached at the 4th day of AF in experiments without chitosan. *T. delbrueckii* showed a delay in the start of AF by 4 days and a V_{max} of 0.69 ± 0.07 g/L h^{-1} (4th day of fermentation) in absence of chitosans, in comparison with a V_{max} of 0.32 ± 0.12 g/L h^{-1} after 9 days in treated experiments. Only *S. cerevisiae* and *S. bayanus,* the latter in the test without chitosans, reached complete sugars consumption; in the other experiments, AF stalled after 20 days of observation, leaving sugars in the medium (Fig. 3B).

MLF were performed considering the three main species of bacteria involved in the degradation of malic acid in wine and cider: *O. oeni*, *L. plantarum* and *P. pentosaceus* (Fig. 3C). In the test performed without chitosans addition *O. oeni* and *L. plantarum* showed the highest rate of malic acid consumption, resulting in the complete degradation of the initial 12 g/L of L-malic acid in less than 4 days. *P. pentosaceus* resulted unable to completely consume L-malic acid in the period of observation, with 6.0 ± 0.5 g/L being left over after 10 days. The addition of a commercial chitosan did not alter the activity of *O. oeni* significantly ($p > 0.05$, One-way ANOVA and Tukey-test), while *L. plantarum*, although with a relevant delay compared to the previous test (p: 1.22 E-04, One-way ANOVA and Tukey-test), accomplished malolactic fermentation in 4 days. The addition of chitosans to the experiments performed with *P. pentosaceus* increased the amount of L-malic acid that remained in the medium at the end of tests (7.4 ± 0.3 g/L).

Sensitivity of *Brettanomyces bruxellensis* to different chitosans

Brettanomyces bruxellensis is the main spoilage agent in oenology, although its use is recommended in mixed fermentation at the basis of production of other fermented beverages, such as at top-fermentation beer [39, 40]. 12 commercial chitosans with different origins and composition were tested against *B. bruxellensis*. Table 4 reports the cell concentration observed after 7 days of incubation of *B. bruxellensis* ATCC 52034 in the presence of chitosans and in constant agitation. Confirming the preliminary results of Table 3, the addition of chitosans seriously impacts on the viability of *B. bruxellensis*, without difference among the various chitosans in terms of dead cells (mean 6.84 ± 0.08 log cell/mL), that represent more than 97% of population. The remaining yeast population, even if it maintained a residual enzymatic activity (FL1 signal), showed a relevant permeabilization of the cells, detectable in both the FL1 and FL2 channels [37]. Table 5 lists the data of the same experiment performed without agitation of synthetic media. The decrease in the total cell concentration as against the previous tests (5.36 ± 0.32 log cell/mL vs. 6.99 ± 0.11 log cell mL, Tables 4 and 5) is attributable to the settling, however, according to the previous experiment, a large prevalence of cell with damaged membrane is observed. Apart from cell settling due to the absence of homogenization, the ANOVA one way test did not reveal differences in the incidence of the dead cell population with respect to the total cell number in the two experiment ($F_{1,20}$: 4.351 p: 0.982).

Residual antimicrobial effect of chitosan after removal

The growth of yeast and bacteria in media already treated with chitosans was essayed to exclude their residual activity after removal. The initial cell load was adjusted at 2 log cell/mL to evaluate the capability of microbes to growth up to sufficient concentrations to activate the bio processes having technological significance. In the majority of experiments dead cells resulted undetectable by flow cytometry (data not reported). All yeasts showed growth up to 7 log units in the medium without chitosan (sample "Test", Table 6); on the contrary, the addition of chitosans of animal origin inhibited yeast's growth for the 4 species of yeast considered. Chitosans of fungal origin appeared less detrimental to yeast, enabling a partial development of *S. cerevisiae*, while *S. bayanus* and *T. delbrueckii* did not grow in the medium previously treated with chitosans. *B. bruxellensis* did not grow in all experiments, despite the

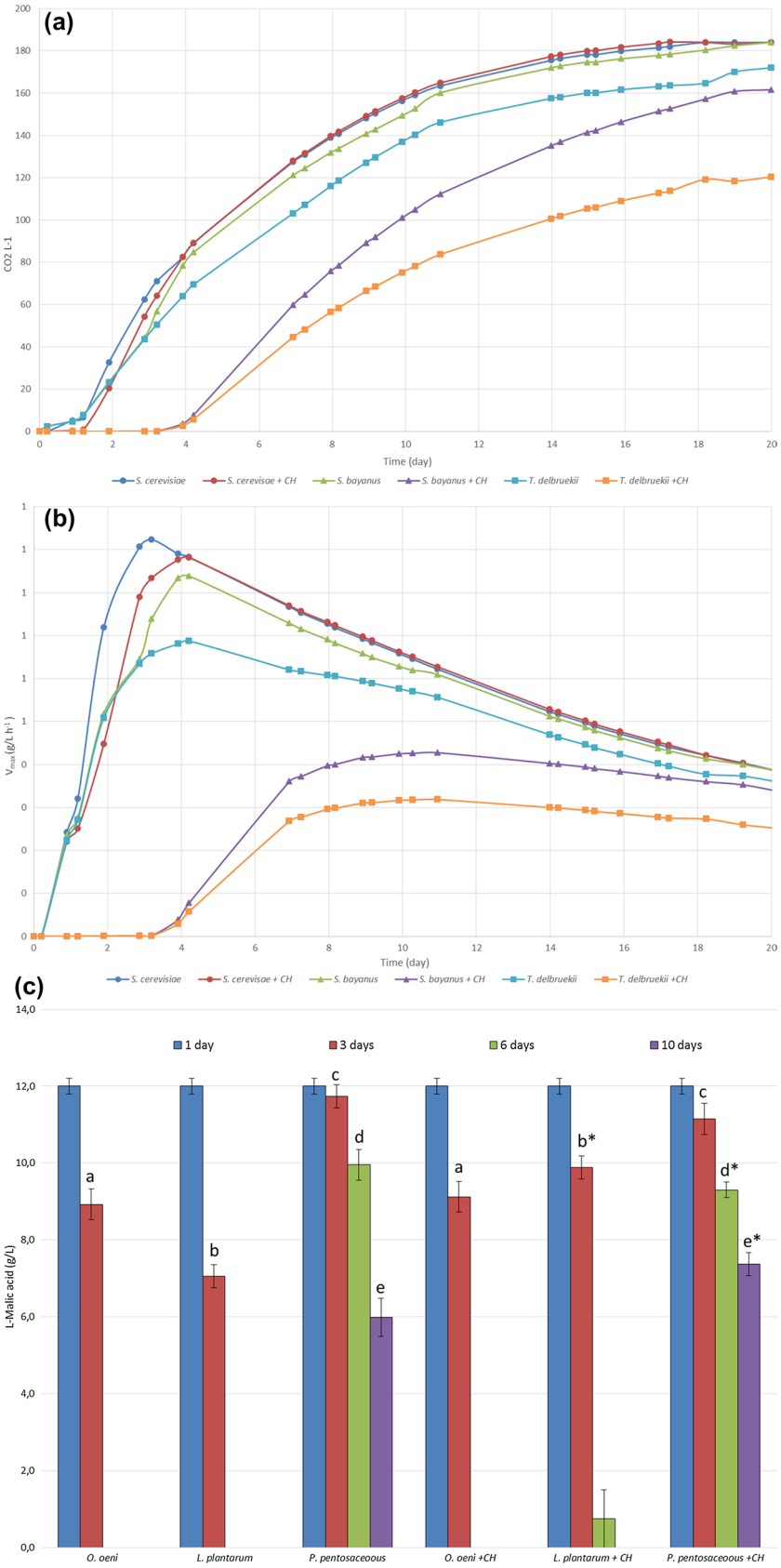

Fig. 3 Experiment of fermentation in presence of chitosan. **A** Behavior of fermentation of the 3 main yeast species involved in the industrial production of alcoholic fermented beverages. Data expressed as weight loss due to CO_2 production (mean data. $n=3$). **B** Evolution of V_{max} of alcoholic fermentation in YM modified medium containing initially 200 g/L of glucose. **C** Degradation of malic acid by 3 main bacteria's specie involved in malolactic fermentation in wine and cider (mean data. $n=3$. Same letter in the apex: data compared by ANOVA one-way + Tukey test. * Data statistically different. p: 0.05)

Table 4 Effect of the addition of different commercial chitosans to a pure culture of *B. bruxellensis* (ATCC 52304. nominal concentration 5.0 log/mL)

Cell concentration in YM after 7 days of incubation at 25 °C Dynamic test					
Chitosan	Origin/composition	Total cell number (FSC)	Damaged cells (FL1/FL2)	Dead cells (FL2)	Ratio FSC/FL2 (%)
	Log cell/mL				
1	Fungi	7.07	5.91	6.95	98.4
2	Animal	7.01	5.67	6.76	96.5
3	Fungi	7.17	5.97	6.94	96.8
4	Animal	6.88	5.70	6.83	99.2
5	Animal	6.85	5.69	6.79	99.2
6	Fungi	7.00	5.87	6.76	96.6
7	Animal	6.99	5.84	6.84	97.8
8	Animal	6.79	5.72	6.72	99.0
9	Fungi	7.05	5.88	6.89	97.7
10	Animal	7.02	5.93	6.96	99.1
11	Fungi	7.10	6.98	6.89	97.1
12	Fungi	6.97	5.64	6.82	97.9
Mean		6.99	5.81	6.85	97.9
DS		0.11	0.12	0.08	1.0
Test without chitosan		6.97	2.68	3.80	7.6

Cellular concentration in YM medium after 7 days of incubation at 25 °C under agitation

Table 5 Effect of the addition of different commercial chitosan to a pure culture of *B. bruxellensis* (ATCC 52304. nominal concentration 5.0 log/mL)

Cell concentration in YM after 7 days of incubation at 25 °C Static test					
Chitosan	Origin/composition	Total cell number (FSC)	Damaged cells (FL1/FL2)	Dead cells (FL2)	Ratio FSC/FL2 (%)
	Log cell/mL				
1	Fungi	5.26	4.34	5.15	97.9
2	Animal	5.52	4.76	5.36	97.2
3	Fungi	5.34	4.49	5.23	97.9
4	Animal	5.48	4.52	5.40	98.6
5	Animal	5.51	4.75	5.40	98.1
6	Fungi	6.04	5.15	5.93	98.2
7	Animal	5.40	4.63	5.30	98.2
8	Animal	4.95	4.00	4.86	98.3
9	Fungi	5.00	4.28	4.89	97.8
10	Animal	5.26	4.28	5.15	97.9
11	Fungi	5.60	4.85	5.46	97.5
12	Fungi	4.92	3.99	4.81	97.7
Mean		5.36	4.50	5.25	97.9
DS		0.32	0.35	0.31	0.4
Test without chitosan		5.94	1.91	2.54	4.0

Cellular concentration in YM medium after 7 days of incubation at 25 °C in static conditions

prolonged incubation time. In the same experiments two species of LAB, *O. oeni* and *L. plantarum* were also tested. The plate count performed after 7 days of inoculation in the medium previously treated with chitosans showed a growth that reached 5.45 ± 0.2 (*O. oeni*) and 5.47 ± 0.2 (*L. plantarum*) log CFU/mL, in both cases lower by two logarithmic orders than the test (Table 6). The one way ANOVA analysis did not reveal significant differences between the results obtained with the two species of bacteria ($F_{1,8}$: 5.317 p: 0.890).

Table 6 Residual inhibition activity of chitosan after removing on insoluble fraction on the microbial growth

Chitosan	Test without chitosan	NB fungi	6 fungi	4 animal	11 Fungi + citric acid (40%)	12 Fungi + bentonite (75%)
Log cell/mL						
Saccharomyces cerevisiae[a]	7.80	3.48	3.91	nd	nd	6.80
Saccharomyces bayanus[a]	7.28	nd	nd	nd	nd	nd
Torulaspora delbrueckii[a]	7.46	nd	2.62	nd	nd	nd
Brettanomyces bruxellensis[b]	7.34	nd	nd	nd	nd	nd
Oenococcus oeni[b]	7.54	5.30	5.53	5.64	5.70	5.08
Lactobacillus plantarum[a]	7.60	5.43	5.79	5.36	5.60	5.18

Test conducted in YM medium (pH 3.50. 7% of glucose. 10% EtOH) having an initial inoculum of 2 log cell/mL. Incubation time:[a]3 days; [b]7 days. Nd.: not detectable (< 2 log units)

Discussion

The use of chitosan as anti-microbial agent is widespread in various agro food fields [11, 14], and hypotheses on the mechanisms of action of these molecules have been advanced by various authors [21, 22]. This work explored the implication of the use of different commercial chitosans in the fermented beverages industry, considering the microorganisms involved in the production of wine, cider and beer, the commercial formulates of chitosan and protocols of treatment similar to those used in the agro-food industries. The chemical characterization of 12 commercial chitosans suggested a way to establish the origin of the raw material of which these molecules are made. This result was achieved by using ionic chromatography analysis on the soluble portions of chitosans, capable to distinguish between the fungal and animal origin of the samples, which is a crucial question to use chitosans that are free of any danger to human safety. Solubility experiments performed in conditions that replicate the typical composition of fermented beverages, with low pH and a relevant ethanol content, indicated that a fraction between 1.5 and 2.0% of each commercial chitosan is soluble, and remains in the beverages after the removal of the insoluble fraction, as required by the protocol of treatments with chitosans. These evidences suggested the need for additional knowledge about the impact of chitosans on the microorganisms that are involved in fermented beverages production.

The first question that must be investigated concerns the effectiveness of chitosans applied to different species of microorganisms of agro-food interest. Previous studies had focused on spoilage or pathogenic microorganisms [39–41], while few data are available regarding the interaction between this polymer and the microorganisms that perform food fermentations. Table 3 shows the microorganisms involved in the study, some of which, such as *Saccharomyces* sp., *T. delbrueckii* and *Oenococcus oeni*, are essential in the production of fermented beverages, as they are responsible for the alcoholic or malolactic fermentation [9]. Acetic bacteria and oxidative yeasts (*Candida* sp., *Pichia* sp.) are sometimes involved in the production of fermented beverages, as well as *Lactobacillus* sp. [42]. *B. bruxellensis*, *S. pombe and S. ludwigii*, apart from some exceptions in the brewing industry, are generally considered spoilage agents [2, 37]. Considering this a preliminary test focused on the different microbial sensitivity at chitosan, we employed a commercial formulate of chitosan having fungal origin, largely diffused in the fermented beverage industry. The effectiveness of the tested commercial chitosan in killing the microbial population appears to depend on the species of microorganism tested (Table 3). In the experiment conducted with constant stirring of the medium, chitosans remained well dispersed and resulted fully active towards microorganisms. In these conditions, many species of yeast did not show viable cells at the end of the test, confirming the remarkable antifungal activity of chitosans, probably favored by the acidity of the medium (pH 3.50), as already observed by Roller and Covill [43]. The antimicrobial effect is particularly relevant when considering that the initial cell load in the medium (7 log/mL) is higher than that usually found in real conditions for many beer and wine spoilage agents [44]. The yeasts having the best fermentative attitude and highest growth rate, *S. cerevisiae* and *T. delbrueckii*, showed a remarkable resistance to chitosan. From the data available in the bibliography, nothing suggests the reason of such higher resistance to chitosans compared to other species [45]. However, it can be surmised that the high growth rate of *S. cerevisiae* and *T. delbrueckii* leads to the development of a viable population starting from a few survivor cells after the contact with chitosans. This hypothesis agrees with the empirical observations, which indicated that chitosans are not effective in case of high microbial contamination. Also, the mechanism of action of chitosans, based on the interaction with some components of the cell wall [17, 46], would favor this explanation, because once it has reacted with the yeasts surface the polymer would be inactivated and, therefore, its antimicrobial effect would decrease proportionally to the density of the yeast population. The different

sensitivity of yeasts to chitosan could be also related to the different composition of the cell surface [47–49]. An hypothesis about the surprisingly difference in the behavior of the two Saccharomyces (*S. cerevisiae* and *S. bayanus*) can be advanced considering the differences in the structure of the cell wall and in the lipid composition and permeability of the membrane of the two yeast [50], which was one of the main site of activity of chitosan [19, 22, 23].

The experiment described in Fig. 2 explores the effect of chitosan against *S. cerevisiae*, considering 16 different strains in the form of ADY widely used in industrial fermentations for the production of alcoholic beverages (Guzzon et al. [51]). The comparison between cell counts in the medium untreated with chitosans in those of dynamic tests shows a reduction in the population of viable yeast cells of at least one logarithmic order, and the consequent increase of the dead cell population. Considering that in flow cytometry death yeasts were identified by the measure of the permeabilization of cell [37], these data confirm the powerful activity of chitosans against external cell structures. The slight increase in live cell population observed in experiments performed without agitation of the medium could be due to the progressive settling of chitosans, with consequent development of yeasts that survive in the medium. The transitory antimicrobial activity of chitosan, in the absence of a constant homogenization, is confirmed by other authors who observed a decline in the bacteriostatic activity of chitosan in an interval of time between 2 and 14 days, according to the chitosan formulation and the nature of the food subject to treatment [26]. The lowest number of dead cells measured in the static test, below the 7 log units/ mL, can be explained by the settling of dead cells which the chitosan has certainly enhanced, but which is commonly observed at the end of AF.

Previous experiments have confirmed the remarkable activity of chitosans towards spoilage microorganisms, but also the sensitivity of some yeasts and bacteria commonly involved in the production of fermented beverages to this polymer. This evidence should not be underestimated, therefore a series of specific experiments were conducted to understand the activity of the chitosans against food fermentations. Figures 3A and 3B show the evolution of CO_2 due to the AF of 3 yeasts involved in beverages production, *S. cerevisiae, S. bayanus* and *T. delbrueckii*. Although in the previous test a remarkable mortality of *S. cerevisiae* was observed in presence of chitosans, its high fermentative vigor means that there is no slowing of AF due to the presence of chitosan, apart from the lengthening of the lag phase due to the initial killing of a part of the yeast population. For the other two yeasts tested, the effect of the addition of chitosans is deleterious. In the test without chitosan addition, the evolution of the AF caused by *S. bayanus* is similar to that of *S. cerevisiae*, the slight delay being due to the different preference of these two yeasts in terms of temperature of fermentation [50]. *T. delbrueckii* showed the lowest fermentation rate and does not consume sugars completely, but this behavior is expected since the role of this yeast in industrial AF is linked to its osmotolerance and secondary enzymatic activity and not to its fermentation efficiency [52]. The addition of chitosan leads to a significant deterioration of the fermentative performances in terms of latency phase, which lasted 3–4 days, and in the V_{max}. The alteration of V_{max} is particularly relevant as, in accordance with the general definition of V_{max}, this parameter defines the best potential activity of the microorganism. Chitosans, therefore, not only eliminate a part of the cells, but alter the metabolism of survived cells. *S. bayanus* and *T. delbrueckii* resulted incapable of completing sugar consumption in presence of chitosan, with a residue of 20 and 40% of sugars, respectively. It can be hypothesized that the alteration of yeast's performances is promoted by both the convective movements caused by AF, that keep the chitosans dispersed in the medium, increasing their effectiveness as we have seen in the dynamic tests, and by ethanol accumulation, that cooperates with chitosans in the alteration of cell permeability.

Figure 3C summarizes the experiments performed to verify the effect of commercial chitosans on the three main species of lactic bacteria of interest in the fermented beverages industry. According to what observed in the case of yeasts, a different sensitivity of the tested species emerges. *Oenococcus oeni* showed no slowdown of malic acid degradation due to the addition of chitosans in the medium. On the contrary, the presence of chitosans altered the behavior of *Lactobacillus plantarum* and *Pediococcus pentosaceus*. The *Lactobacillus* strain completed the MLF in the presence of chitosans with a delay of some days as against the test while, in the case of *Pediococcus* some malic acid remains at the end of experiment. With regard to what was observed in the case of yeasts, the growth rate appears to be unrelated to chitosan sensitivity, since of the three species *O. oeni* it was certainly the slowest in development. On the contrary, the efficiency in MLF would seem to correlate positively to the resistance to chitosan action; this could be related to the alterations that the polymer causes in the bacterial membrane, since MLF is strictly based on the balance between cytoplasm and the environment, mediated by membrane transporters. Beyond the hypotheses on the resistance mechanism, the result of this test is certainly of interest because chitosans could prevent the alteration of fermented beverages, frequently induced by lactobacilli and pediococci [5] without inhibiting MLF performed by *O. oeni*. Tables 3 and 4 further analyze the effect of various chitosans on *B. bruxellensis*. The differences observed in the numbers of cells in the dynamic and static experiments are due to the settling of cells after a few days, because the ratios among the existing populations are constant in both experiments. *B. bruxellensis* appears particularity affected

by the addition of chitosan, independently form its origin and chemical features. In both cases the dead cells are over the 96% of the entire population; additionally, the cells having residual metabolic activity reveal cell membrane damage at flow cytometry analysis. However, is possible to confirm that chitosan is an effective defense against *B. bruxellensis*.

The experiment of Table 5 investigates the effect of the soluble portion of chitosan on microorganisms, to understand if a treatment with chitosans in the early stages of the fermented beverage production can interfere with fermentation, such as in the secondary AF necessary in the production of sparkling beverages or in MLF. This aspect is crucial considering the alteration in yeast/bacteria metabolisms observed in previous experiments. In is also reasonable that the soluble portion of chitosan is the highest active against microorganisms because its low molecular weight and therefore high capability to penetrates inside cell wall. Another point to consider is whether chitosans can guarantee the long-term protection of beverages, like other preservative agents (sorbate, SO_2, etc..). The evaluation of chitosan solubility in beverage conditions confirmed that a portion equal to a maximum of 2% of the initially added chitosans dissolves stably in an alcoholic solution at pH 3.50. The presence of this residual amount of chitosan has a remarkable efficacy towards the fermentative activity of all yeast species considered, with the exception of some commercial formulates of chitosan made by fungi that allow the development, albeit modest, of *S. cerevisiae* and *T. delbrueckii*. Probably, the initial low cell load amplified the effect of chitosans, reducing the capability of yeast to develop, reaching a concentration sufficient to start AF. This effect must be careful considered, in particular in the case of AF made by microflora native of beverage raw materials, as frequently observed in traditional beverages of many country of word. For bacteria, in accordance with previous tests, the effect of chitosans although evident, is not as great as that of yeast, given that the population decreases by about two orders of magnitude. Certainly, the residual microbial populations observed can cause, in appropriate conditions, a redevelopment and therefore it is not possible to say that chitosans are able to guarantee long-term microbial stability. However, their early use, before the conclusion of the alcoholic and malolactic fermentations, and in particular on the raw materials, must be carefully considered, so as not to encounter problems such as fermentation stalling. Further studies under real conditions, i.e. using grape must or other raw materials, will be necessary to confirm the complex interactions between chitosans and food-related microorganisms that this study has brought to light.

In conclusion, chitosans are a promising agent for an effective control of spoilage agents that affect fermented beverages. They also contribute to the enhancement of the safety of these beverages, because they reduce the accumulation of toxic compounds, such as biogenic ammines, due to spoilage microorganisms activity, with a minimal residing in the finished beverages, since almost 98% of chitosan was easily removed from the medium after treatment. However, in the present study, the interference of chitosan with both microorganisms involved in the fermented beverages production was proven, underscoring the need for a careful use of this polymer, in particular in the early stages of the beverage production process. The most efficacy of chitosan was observed in a medium in constant agitation; this observation overshadows the physical separation of microorganisms from the food matrix caused by chitosans. The use of flow cytometry has confirmed the alterations induced by chitosans in cellular permeability, which therefore remains the main mechanism of action of this molecule. In conclusion, chitosans are a powerful control tool against microbial alterations in beverages; a careful validation towards the microbial species of interest and in the specific conditions of use is necessary to make the most of their potential activities.

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Declarations

Conflict of interest The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Compliance with ethics requirements This article does not contain any studies involving human participants or animals performed by any of the authors.

References

1. Nguyenthe C, Carlin F (1994) The microbiology of minimally processed fresh fruits and vegetables. Crit Rev Food Sci Nutr 34:371–401
2. Fleet G (1992) Spoilage Yeasts. Crit Rev Biotechnol 12:1–44
3. Danilewicz JC (2007) Interaction of sulfur dioxide, polyphenols, and oxygen in a wine-model system: central role of iron and copper. Am J Enol Vitic 58:53–60
4. Henick-Kling T, Edinger W, Daniel P, Monk P (1998) Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J App Microbiol 84:865–876
5. Bartowsky EJ (2009) Bacterial spoilage of wine and approaches to minimize it. Lett Appl Microbiol 48:149–156

6. Macris BJ, Markakis P (1974) Transport and toxicity of sulfur dioxide in *Saccharomyces cerevisiae* var. *ellipsoideus* (view record in Medline). J Sci Food Agric 25:21–29
7. Lambert RJ, Stratford M (1999) Weak-acid preservatives: modelling microbial inhibition and response. J App Microbiol 86:157–164
8. Carrete R, Vidal MT, Bordons A, Constanti M (2002) Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. FEMS Microbiol Lett 211:155–159
9. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D (2007) Handbook of enology, volume 2: the chemistry of wine-stabilization and treatments, 2nd edn. Wiley, Chichester
10. Du Toit WJ, Pretorius LS, Lonvaud-Funel A (2005) The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. J App Microbiol 98:862–871
11. Gomez-Rivas L, Escudero-Abarca BI, Aguilar-Uscanga MG, Hayward-Jones PM, Mendoza P, Ramirez M (2004) Selective antimicrobial action of chitosan against spoilage yeasts in mixed culture fermentations. J Ind Microbiol Biotechnol 31:16–22
12. Han LK, Kimura Y, Okuda H (1999) Reduction in fat storage during chitin-chitosan treatment in mice fed a high-fat diet. Int J Obes 23:174–179
13. Felt O, Buri P, Gurny, (1998) Chitosan: a unique polysaccharide for drug delivery. Drug Dev Ind Pharm 24:979–993
14. Bautista-Baños S, Romanazzi G, Jiménez-Aparicio A (2016) Chitosan in the preservation of agricultural commodities. Academic Press, Cambridge
15. Kumar S, Ye F, Dobretsov S, Dutta J (2019) Chitosan nanocomposite coatings for food, paints, and water treatment applications. Appl Sci 9:2409
16. Chinnici F, Natali N, Riponi C (2014) Efficacy of chitosan in inhibiting the oxidation of (+)catechin in white wine model solutions. J Agric Food Chem 62:9868–9875
17. Cheung R, Ng T, Wong J, Chan W (2015) Chitosan: an update on potential biomedical and pharmaceutical applications. Mar Drugs 13:5156–5186
18. Taillandier P, Joannis-Cassan C, Jentzer JB, Gautier S, Sieczkowski N, Granes D, Brandam C (2015) Effect of a fungal chitosan preparation on *Brettanomyces bruxellensis*, a wine contaminant. J App Microbiol 118:123–131
19. Mandon P, Prasad E (2020) Chitosan market by source (shrimp, squid, crab, krill, and others) and application (water treatment, biomedical and pharmaceutical, cosmetics, food & beverage, and others): global opportunity analysis and industry forecast, 2020–2027. Adv Mat A 01409:231
20. Vaara M (1992) Agents that increase the permeability of the outer-membrane. Microbiol Rev 56:395–411
21. Wang GH (1992) Inhibition and inactivation of 5 species of food-borne pathogens by chitosan. J Food Prot 55:916–919
22. Tsai GJ, Su WH (1999) Antibacterial activity of shrimp chitosan against *Escherichia coli*. J Food Prot 62:239–243
23. Devlieghere F, Vermeulen A, Debevere J (2004) Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiol 21:703–714
24. Strand SP, Vandvik MS, Varum KM, Ostgaard K (2001) Screening of chitosans and conditions for bacterial flocculation. Biomacromol 2:126–133
25. Allan CR, Hadwiger LA (1979) Fungicidal effect of chitosan on fungi of varying cell-wall composition. Exp Mycol 3:285–287
26. Roller S, Covill N (1999) The antifungal properties of chitosan in laboratory media and apple juice. Int J Food Microbiol 47:67–77
27. Marquez IG, Akuaku J, Cruz I, Cheetham J, Golshani A, Smith ML (2013) Disruption of protein synthesis as antifungal mode of action by chitosan. Int J Food Microbiol 164:108–112
28. Ranaldi G, Marigliano I, Vespignani I, Perozzi G, Sambuy Y (2002) The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line. J Nut Biochem 13:157–167
29. OIV (2019) Resolution E-COEI-1-CHITOS, ISBN:978-28-50380-01-3. OIV, Paris
30. Trotel-Aziz P, Couderchet M, Vernet G, Aziz A (2006) Chitosan stimulates defense reactions in grapevine leaves and inhibits development of *Botrytis cinerea*. Eu J Plant Pathol 114:405–413
31. Vitalini S, Gardana C, Zanzotto A, Fico G, Faoro F, Simonetti P, Iriti M (2011) From vineyard to glass: agrochemicals enhance the melatonin and total polyphenol contents and antiradical activity of red wines. J Pineal Res 51:278–285
32. Malacarne M, Nardin T, Bertoldi D, Nicolini G, Larcher R (2016) Verifying the botanical authenticity of commercial tannins through sugars and simple phenols profiles. Food Chem 206:274–283
33. Rinaudo M, Pavlov G, Desbrieres J (1999) Influenced of acetic acid concentration on the solubilization of chitosan. Polymer 40:7029–7032
34. Chen RH, Hwa H (1996) Effects of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical, and permeability properties of the prepared membrane. Carbohyd Polym 29:353–358
35. OIV (2020) Compendium of international methods of wine and must analysis. OIV-MA-AS313–11. OIV, Paris
36. Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek Int J Gen Mol Microbiol 73:331–371
37. Guzzon R, Larcher R (2015) The application of flow cytometry in microbiological monitoring during winemaking: two case studies. Ann Microbiol 65:1865–1878
38. OIV (2020) OIV F-COEI-2-CONBAC 9. OIV, Paris
39. Ardila N, Daigle F, Heuzey MC, Ajji A (2017) Effect of chitosan physical form on its antibacterial activity against pathogenic bacteria. J Food Sci 82:679–686
40. Guzzon R, Larcher R, Guarcello R, Francesca N, Settanni L, Moschetti G (2018) Spoilage potential of *Brettanomyces bruxellensis* strains isolated from Italian wines. Food Res Int 105:668–677
41. Karsli B, Caglak E, Li DP, Rubio NK, Janes M, Prinyawiwatkul W (2018) Inhibition of selected pathogens inoculated on the surface of catfish fillets by high molecular weight chitosan coating. Int J Food Sci Technol 54:25–33
42. Jay JM, Loessner MJ, Golden DA (2006) Modern food microbiology. Springer-Verlag Inc., New York
43. Roller S, Covill N (2000) The antimicrobial properties of chitosan in mayonnaise and mayonnaise-based shrimp salads. J Food Prot 63:202–209
44. Guzzon R, Bernard M, Barnaba C, Bertoldi D, Pixner K, Larcher R (2017) The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine. J Food Sci Technol Mysore 54:810–821
45. Rajoka MSR, Mehwish HM, Wu YG, Zhao LQ, Arfat Y, Majeed K, Anwaar S (2020) Chitin/chitosan derivatives and their interactions with microorganisms: a comprehensive review and future perspectives. Crit Rev Biotechnol 40:365–379
46. Rabea EL, Badawy ME, Stevens CV, Smagghe G, Steurbaut W (2003) Chitosan as antimicrobial agent: applications and mode of action. Biomacromol 4:1457–1465

47. Pena A, Sanchez NS, Calahorra M (2013) Effects of chitosan on *Candida albicans*: conditions for its antifungal activity. Biomed Res Int 2013:1–16. https://doi.org/10.1155/2013/527549
48. Park Y, Kim MH, Park SC, Cheong H, Jang MK, Nah JW, Hahm KS (2008) Investigation of the antifungal activity and mechanism of action of LMWS-Chitosan. J Microbiol Biotechnol 18:1729–1734
49. Palma-Guerrero J, Lopez-Jimenez JA, Perez-Berna AJ, Huang IC, Jansson HB, Salinas J, Villalain J, Read ND, Lopez-Liorca LV (2010) Membrane fluidity determines sensitivity of filamentous fungi to chitosan. Mol Microbiol 75:1021–1032
50. Torija MJ, Beltran G, Novo M, Poblet M, Guillamon JM, Mas A, Rozes N (2003) Effects of fermentation temperature and *Saccharomyces* species on the cell fatty acid composition and presence of volatile compounds in wine. Int J Food Microbiol 85:127–136
51. Guzzon R, Nicolini G, Nardin T, Malacarne M, Larcher R (2014) Survey about the microbiological features, the oenological performance and the influence on the character of wine of active dry yeast employed as starters of wine fermentation. Int J Food Sci Technol 49:2142–2148
52. Bely M, Stoeckle P, Masneuf-Pomarède I, Dubourdieu D (2008) Impact of mixed *Torulaspora delbrueckii Saccharomyces cerevisiae* culture on high-sugar fermentation. Int J Food Microbiol 122:312–320