Impact of Spray-Drying on Biological Properties of Chitosan Matrices Supplemented with Antioxidant Fungal Extracts for Wine Applications

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https://doi.org/10.1007/s00284-019-01804-7

Impact of Spray-Drying on Biological Properties of Chitosan Matrices Supplemented with Antioxidant Fungal Extracts for Wine Applications

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Abstract
Black aspergilli produce many bioactive compounds: enzymes, organic acids, and secondary metabolites. One such fungus, *Aspergillus tubingensis* G131, isolated from French Mediterranean vineyards, produces secondary metabolites with antioxidant properties that can be extracted with ethanol. In this study, crude antioxidant extracts obtained from *A. tubingensis* G131 cultures were encapsulated with two types of chitosan matrix. Spray-drying was used to obtain dried particles from a dispersion of fungal crude extracts in a solution of the coating agent chitosan. This process appeared to be an efficient method for obtaining a dry extract with antioxidant activity. Three types of fungal extracts, with different antioxidant capacities, were produced: two different concentrations of crude extract and a semi-purified extract. In this study, the chitosan matrices for encapsulation were chosen on the basis of their antimicrobial activities for wine applications. Classical low molecular weight chitosan was compared with NoBrett Inside® which is already used to prevent the development of *Brettanomyces* spp. in wine. The objective of this study was to confirm that both antioxidant (fungal extract) and antimicrobial (chitosan) properties were preserved after spray-drying. The combination of these two properties and the powder formulation of this entirely natural product would make it a good alternative to chemicals, such as sulfites, in the food and wine industries.

Keywords Fungal metabolites · Chitosan · Microencapsulation · Spray-drying · Antioxidant

Abbreviations

ABTS	2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid
AE	Additive effect
AN	Antagonistic effect
CE	Crude extract
LMW	Low molecular weight
NBI	No Brett inside®
NGPs	Naphtho-gamma-pyrones
PE	Semi-purified extract
RT	Room temperature
SPE	Solid-phase extraction
SE	Synergistic effect
SEM	Scanning electron microscopy
TEAC	Trolox equivalent antioxidant capacity

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Introduction

One of the key challenges in the wine industry is controlling wine stability during production, aging, and after bottling. During wine aging, the principal objectives of the winemaker are preventing wine spoilage and stabilizing wine color by limiting oxidation. Sulfites are added at various steps in the winemaking process to achieve these objectives. However, it has been suggested that sulfites may have adverse effects on human health, such as pseudoallergies, and winemakers are therefore now trying to limit the use of sulfites in the winemaking process [1–3]. Indeed, wine consumers prefer high-quality less-processed wines [4, 5]. For winemakers to meet this demand, the development of new preservative agents or stabilization techniques is required. Natural products are currently defined as metabolites produced by living organisms and/or naturally occurring in nature. Such metabolites from various organisms are capable of preventing microbial

spoilage, and thus are a suitable alternative to the use of synthetic products [6]. Among them, chitosan meets these requirements, and the European Union (EU) and International Organization of Vine and Wine (OIV) have already approved its use for wine applications. The OIV/OENO 338A/2009 (International Organization of Vine and Wine (OIV) 2009) resolution added the use of chitosan in winemaking to the International Code of Oenological Practice, specifying that the maximum dose of chitosan used to reduce ochratoxin A (OTA) levels must not exceed 500 g/hL. Chitosan is a linear polysaccharide composed of two repeating units (D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GLcNAc)) randomly distributed along the polymer chain and linked by β(1-4)-bonds. Various activities have been demonstrated for different types of chitosan: as a fining and protein stabilizing agent, a preservative and an antimicrobial agent [3, 7, 8]. Chitosan has antimicrobial effects against lactic acid bacteria, acetic acid bacteria, fungi, and undesirable yeasts, such as *Brettanomyces* sp., during aging, but it is permissive for the growth of *Saccharomyces* species [7, 9–12].

For wine aging applications, it would be useful to couple the antimicrobial properties of chitosan with antioxidant activity to stabilize wine color. In this study, an ethanol extract of *Aspergillus tubingensis* G131 was chosen. This black Aspergilli strain was isolated during a survey on the occurrence of OTA producers on grapes from various French vineyards [13]. *A. tubingensis* G131, isolated from a French Mediterranean Vineyard, does not produce OTA [14]. An ethanol extract of a sporulated mycelial cake from this strain was found to be dark gold in color, due to the presence of melanin, and has interesting antioxidant properties. Equivalent extracts from other black Aspergilli strains have already been reported to have antioxidant activity. For example, a methanol extract from an *A. niger* strain was found to have beneficial effects on rat growth and hepatoprotective activity [15]. Another organic extract of *A. niger* was shown to prevent lard oxidation [16]. These findings suggest that organic extracts of non-mycotoxigenic black Aspergilli are of potential interest for their use as preservatives in the food industry.

A suitable strategy for efficiently combining the antimicrobial properties of chitosan with the antioxidant activity of the chosen fungal extract was therefore required. Microencapsulation appeared to be an effective approach.

Interest in antioxidant encapsulation has recently increased, particularly in the food industry. This process makes it possible to increase the stability of bioactive compounds during processing and storage and prevents undesirable interactions with the other components of a formulation [17–19]. The protective mechanism of microencapsulation involves the formation of a membrane wall to enclose droplets or particles of the encapsulated ingredient, thereby protecting it, improving its stability, and making it possible to convert liquid preparations into powders, or to improve their dispersibility in water [20].

Various microencapsulation technologies, such as spray-drying, prilling, coacervation, extrusion, and in situ polymerization, are available. Spray-drying is one of the simplest and most widely used processes due to its relatively low cost and efficiency and the availability of appropriate industrial equipment [21]. Antioxidant encapsulation by the spray-drying method has already been described for various wall materials, such as plant proteins [22–24], milk proteins [25], various carbohydrates, including maltodextrins [26–30], and blends of maltodextrin/gum arabic [31], or maltodextrin/K-carrageenan [32], and for chitosan [33–35].

The objectives of this work were to study the antimicrobial and antioxidant capacities of chitosan/fungal extract microparticles obtained by spray-drying for potential applications in the food industry, including the maintenance of wine stability. Indeed, the combination of these two types of activity should prevent both the spoilage and oxidation of the wine. Microparticles of this type will not only combine two biological properties, but also will incorporate into food in solid form, without dissolution, thereby preventing alterations to organoleptic qualities, such as color [35]. The chitosan/fungal extract microparticles were subjected to physicochemical (particle size, morphology and moisture) and biological (impact on yeasts growth, antioxidant capacity) characterization.

Materials and Methods

Chemicals

Chitosan 1 (NBI)

This chitosan preparation was a powder with particles of less than 50 µm in diameter, and a product of the deacetylation of chitin extracted from *A. niger* supplied by KitoZyme company (Herstal, Belgium): KiOfine B® or No Brett Inside® (commercially available products). This product was less than 30% acetylated and the viscosity of a 1% solution in acetic acid was about 4 mPa.s.

Chitosan 2 (LMW)

The chitosan preparation was obtained from Glentham Life Sciences (Wiltshire, United Kingdom) and used without purification. It had a very low molecular weight (average molecular weight: 30,000 g mol^{-1}) and was less than 10% acetylated.

These two chitosans had low molecular weights and acetylation levels, features known to be associated with the antibacterial activity of chitosan [36].

The anhydrous ethanol (Fisher®), acetic acid, and other chemicals (Sigma-Aldrich®) used were of analytical grade.

Fungal Extracts

Production of the Crude Fungal Extract

Czapek Yeast Broth (CYB—30 g/L Saccharose, 5 g/L Yeast Extract, 2 g/L $NaNO_3$, 0.25 g/L KCl, 0.25 g/L $MgSO_4.7H_2O$, 0.005 g/L $FeSO_4.7H_2O$, 0.5 g/L K_2HPO_4, 0.001 g/L $ZnSO_4.7H_2O$ and 0.0005 g/L $CuSO_4.7H_2O$) was inoculated with 10^7 spores of *A. tubingensis* G131, and the resulting fungal cultures were incubated for 7 days at 30 °C. The mycelial cake was then separated from the culture medium. The mycelial cake only was then covered with anhydrous ethanol. The mycelium/ethanol mixture was incubated for 20 min at room temperature (RT) and then subjected to sonication for 20 min at 50 Hz. The sonicated mycelium/ethanol mixture was filtered once through 113 V grade Whatman filter paper. The filtered extract obtained was stored in the dark at 4 °C until required, and is referred here as the crude extract (CE).

Concentration by Rotary Evaporation

The crude extract was concentrated (volume to volume) with a rotary evaporator. The conditions for evaporation were as follows : 60 °C, rotation at 150 rpm, and uncontrolled vacuum and cooling with distilled tap water at 15 °C. Crude extracts (CEs) were named on the basis of their concentration yield. In this study, two concentrated extracts were used: CE*3 (3 V/V: 3 volumes were evaporated in 1 remaining volume) and CE*6 (6 V/V: 6 volumes were evaporated in 1 remaining volume).

Solid-Phase Extraction

Solid-phase extraction (SPE) was performed on a C18 Hypersep column (Thermo Fischer Scientific)—Bed weight: 5 g; Sorbent: Hypersep C18; Particle Size: 40–60 µm; and column capacity: 25 mL. Columns were equilibrated with five volumes of acetonitrile and three volumes of MilliQ water. 5 mL of CE*3 was deposited on the column and washed with two volumes of water/acetonitrile (70:30 mixture). Finally, a semi-purified fraction was eluted in one volume of anhydrous ethanol. This semi-purified SPE product was then concentrated by rotary evaporation (2 V/V: 2 volumes were evaporated in 1 remaining volume) and named (PE*2).

Estimation of the Dry Weight of Fungal Extracts

The dry weight of all the fungal extracts obtained was determined with an EM120-HR Moisture Analyzer (Precisa Gravimetrics AG, Dietikon, Switzerland) by drying to constant weight at 70 °C.

HPLC Characterisation of the Fungal Extracts

HPLC analyses were performed as described in Choque et al. [14].

Microencapsulation Methodology

Preparation of the Solution

Solution of 1% (w/w) chitosan in 1% (v/v) aqueous acetic acid was prepared with mechanical stirring (500 rpm) at RT. Fungal extracts in ethanol (CE*3, CE*6, and PE*2) were then added to the chitosan solution, 80% of chitosan solution and 20% of fungal extract, with mechanical stirring (500 rpm) to mix them before the spray-drying process.

Preparation of Spray-Dried Microparticles

Obtained chitosan/fungal extract solutions were spray-dried in a Mini Spray Dryer B-290 (Büchi, Flawil, Switzerland) in the open mode under the following conditions: inlet air temperature at 120 ± 4 °C and outlet temperature at 75 ± 4 °C, drying air flow rate of 470 L/h, liquid feed flow rate varying from 0.33 to 0.48 L/h and 100% aspiration. Microparticles were collected from the container, sealed hermetically in an opaque packaging, and stored at room temperature.

Spray-drying yield was calculated as follows:

$$\text{Spray} - \text{drying yield}\ (\%) = M_P/M_{RM} \times 100$$

where M_P is the dry mass of the microparticles collected and M_{RM} is the initial dry mass of the solids in the solution of chitosan and fungal extract.

Physicochemical Characterization of Microencapsulated Matrices

Moisture Content

The moisture content of all the powders obtained was determined with an EM120-HR Moisture Analyzer (Precisa Gravimetrics AG, Dietikon, Swiss) by drying to constant weight at 105 °C.

Microparticles Size Distribution

The particle size distribution of dried microparticles was determined from the scattering pattern of the transverse laser light with a Scirocco 2000 instrument (Malvern Instruments, Worcestershire, UK). Mean particle diameter ranged from 0.2 to 2000 µm. The following parameters were used: refractive index of 1.52, pressure of air of dispersion of 4 bars, degree of vibration of 70%. The volume-based particle diameter (D_{43} or D_v) was calculated as the mean of three measurements per sample.

Microparticle Microstructure

The morphology of microparticles was examined by scanning electron microscopy (SEM). The particles were deposited on conductive double-faced adhesive tape and sputter-coated with silver. The microparticles were frozen in liquid nitrogen and broken up in a mortar, for examination of their internal structure. SEM observations were performed with a LEO435VP scanning electron microscope (LEO Electron Microscopy Ltd., Cambridge, UK) operating at 8 kV.

Microencapsulated Matrix Suspension

Each microencapsulated matrix was dissolved in acidified MilliQ water (pH 3.0, acetic acid), 12% ethanol (pH3.0, acetic acid) or 100% ethanol to give a 3 g/L (dry weight) solution. The weighted powder was incubated in the appropriate solvent for 24 h at RT and then stored at 4 °C. Each experiment was run in triplicate.

Effect of Microencapsulated Matrices on Enological Yeasts Growth

The effects of spray-drying chitosan and its combination with a fungal antioxidant on growth of two enological strains—*Saccharomyces cerevisiae* and *Brettanomyces bruxellensis*—were assessed.

Yeasts were precultured in YEPD (10 g/L yeast extract, 20 g/L meat peptone, 20 g/L D-glucose) for 16 h at 30 °C. Two concentrations of chitosan were used, based on the results of Taillandier et al. (Taillandier et al., 2015), describing the mode of action of NBI chitosan on *B. bruxellensis* inhibition: 40 mg/L and 400 mg/L [11]. These concentrations were chosen as they correspond to the ends of the standard range of NBI showing an action on *B. bruxellensis*. The controls were culture media without any supplementation, and culture media supplemented with low molecular weight chitosan or with No Brett Inside chitosan that had not been spray-dried. The supplements to the YEPD medium were added 16 h before inoculation, and the medium was allowed to equilibrate at 20 °C. The YEPD medium was then inoculated to an OD_{600nm} of 0.1 with yeast preculture. The growth of the yeasts was monitored by spectrophotometry at $\lambda = 600$ nm,at times 0, 8 h, 24 h, and 48 h. OD was also estimated before inoculation to ensure the correct estimation of yeast growth. The experiments were carried out in duplicate, at 20 °C, and flasks were stirred just after inoculation and immediately before sampling.

Trolox Equivalent Antioxidant Capacity

The antioxidant activity of the fungal extracts was determined in the TEAC test, according to a protocol adapted from a previous study [37]. $ABTS^+$ was produced by the reaction of 7 mM of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (Sigma-Aldrich, > 99% HPLC grade) with 2.5 mM of $K_2S_2O_8$ (sodium persulfate) (Sigma-Aldrich, > 99% ACS reagent) in distilled water for 16 h at RT, in the dark. The concentration of $ABTS^+$ was then adjusted by dilution to an absorbance of 0.7 ± 0.02 (at 734 nm) on a spectrophotometer (Anthelie advanced, SECONAM). A stock solution at 150 mM was prepared for Trolox (Sigma-Aldrich). A 100 µL aliquot of the desired sample, at the appropriate dilution, or the Trolox standard (final concentration of 2.5, 5, 10 or 15 µM) was added to 900 µLl of the diluted $ABTS^+$. Absorbance was measured at 734 nm after incubation in the dark, at RT, for 6 min. Each sample was tested three times in duplicate. TEAC value was estimated in mM as described by Re et al. [37].

Classification of Additive, Synergistic or Antagonistic Effects

As described by Ribeiro et al. [38], theoretical values for the antioxidant activity of the assayed extract mixture were calculated as the weighted mean of the experimentally determined TEAC values of the individual extracts. For example, for CE*3, the estimated TEAC was based on the dilution factor applied to both the chitosan and fungal extracts in the microencapsulated matrix suspension. It was therefore calculated as follows:

$$\mathrm{TEAC}_{\mathrm{est}} = \left(\mathrm{TEAC}_{\mathrm{fungal\ extract}} \times \left(M_{\mathrm{fe-c}} / M_{\mathrm{fe-i}}\right)\right) + \left(\mathrm{TEAC}_{\mathrm{chitosan}} \times \left(M_{\mathrm{c-c}} / M_{\mathrm{c-i}}\right)\right)$$

where $M_{\mathrm{fe\text{-}c}}$ is the dry mass of fungal extract in the microencapsulated matrix suspension; $M_{\mathrm{fe\text{-}i}}$ is the dry mass of the initial fungal extract; $M_{\mathrm{c\text{-}c}}$ is the dry mass of chitosan in the microencapsulated matrix suspension; and $M_{\mathrm{c\text{-}i}}$ is the dry mass of the initial chitosan solution.

Effects were classified as additive (AE), synergistic (SE) or antagonistic (AN) as described by Ribeiro et al. [38]. AE: theoretical and experimental values differed by less than 5%. SE: experimental values were at least 5% higher than theoretical values. AN: experimental values were at least 5% lower than theoretical values. Lower TEAC values are associated with lower levels of antioxidant activity.

Statistical Analyses

Data are presented as means ± SD for triplicate experiments. Statistical analyses were performed with jamovi (jamovi project (2017), jamovi (Version 0.8)). One-way and two-way ANOVA tests were used to assess the significance of differences for each variable ($p < 0.05$). Tukey post hoc test was applied to determine the significance of differences between conditions.

Results

Microencapsulation of Fungal Extracts

Spray-drying was used to prepare chitosan and ethanol extract-loaded chitosan microparticles. Several parameters can affect the characteristics of the obtained microspheres, including polymer concentration, solvent and assay conditions (inlet temperature, spray rate of feed, etc.) [39].

For these experiments, chitosan concentration was fixed at 1% (w/w) in 1% (v/v) aqueous acetic acid to ensure complete dissolution of the chitosan and solutions with a suitable viscosity for spraying. Ethanol extracts (CE*3, CE*6, PE*2, 100 mL in each case) were then added to give an 80/20 water/ethanol ratio, which maintained suitable levels of solubility of both chitosan and the extracts in the solution, preventing obstruction of the nozzle during the spray-drying process and increasing encapsulation efficiency [21, 40].

For the spray-drying parameters, the chosen inlet temperature of 120 °C was sufficient to evaporate both solvents and acetic acid (which has a boiling point is 118 °C), and the feed spray rate was adapted to maintain the outlet temperature around 75 °C, ensuring that the particle dried correctly with no alteration of the properties of the extracts. Appropriate adjustment between the inlet temperature and the feed flow is essential to ensure the evaporation of maximum of the liquid sprayed before the droplets meet the drying chamber walls, to optimize the production yield [40].

The incorporation of ethanol extract, even with a high proportion of fungal material (PE*2: 1.39 ± 0.03 g/L; CE*3: 29.4 ± 0.7 g/L and CE*6: 55.1 ± 1.2 g/L (dry weight)), made it possible to obtain production yields of around 80% (Table 1). These results were highly satisfactory and significantly better than published production yields for the use of chitosan as the encapsulation material, which vary from 23 to 73%, and are generally around 50% [21, 41].

Mean particle size ranged from 1.17 to 4.35 µm, consistent with published values for chitosan and loaded chitosan microparticles, which are generally between 1 and 5 µm [21]. The results of the statistical analysis suggested that mean particle size depends on the type of fungal extract used for supplementation, but not of the type of chitosan. These values were confirmed by SEM observations (Fig. 1). This could be linked to the composition of the extract. HPLC analysis of the extract were performed as described in Choque et al. [14]. Results show that fungal extracts are composed of two main compounds: melanin (black pigment), and Naphtho-Gamma-Pyrones (NGPs). The proportion of NGPs of each extract was calculated with chromatogram analysis. The proportions were 94.4 ± 2.3% of NGPs

Table 1 Spray-drying yield and mean micro particle diameter

Chitosan	Fungal extract	Initial dry weight (g)	Spray-drying yield (%)	Mean microparticle size (µm)	Particle size coefficient of variation (%)
LMW	None	3.998	61	1.52 ± 0.03[a]	1.64
	PE*2	4.100	81	2.49 ± 0.01[b]	0.40
	CE*3	6.944	80	3.29 ± 0.04[c]	1.09
	CE*6	9.497	81	4.35 ± 0.33[d]	7.59
NBI	None	4.054	78	1.17 ± 0.05[a]	4.10
	PE*2	4.170	74	2.26 ± 0.01[b]	0.35
	CE*3	6.961	80	3.13 ± 0.01[c]	0.10
	CE*6	9.492	79	3.37 ± 0.14[c]	4.24

LMW low molecular weight chitosan, *NBI* No Brett Inside® chitosan; *None* anhydrous ethanol control, *PE*2* SPE semi-purified fungal extract, *CE*3* fungal extract concentrated by rotary evaporation (3 V/V), *CE*6* fungal extract concentrated by rotary evaporation (6 V/V)

Data with the same letter are not significantly different (two-way ANOVA with Tukey post hoc correction, p-value < 0.05)

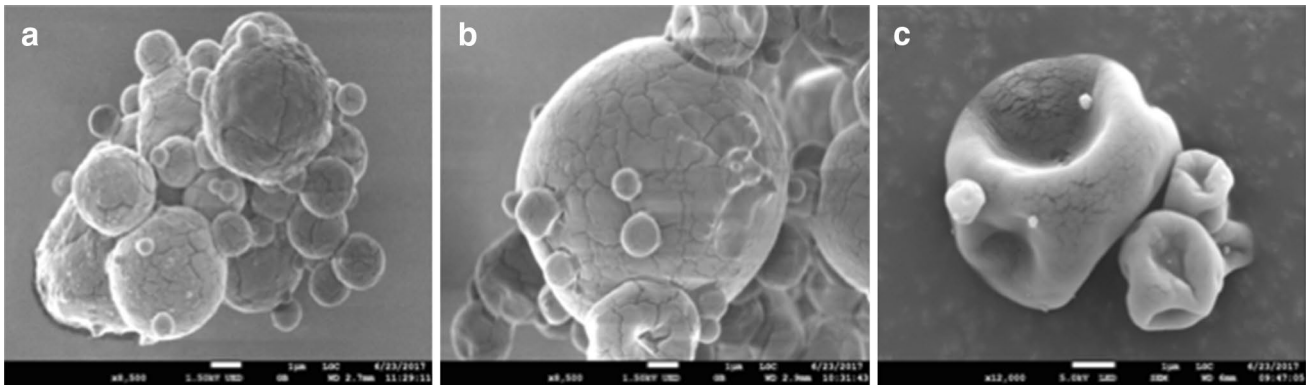

Fig. 1 Scanning electron micrographs of LMW + CE*6 microparticles (**a**), NBI + CE*6 microparticles (**b**), and NBI + None microparticles (**c**)

in PE*2; 34.2 ± 2.5% of NGPs in CE*3, and 28.3 ± 1.8% of NGPs in CE*6. The more the NGPs are present, the less the melanin is. Thus, it appears that the particle size of the chitosan microparticles is linked to the proportion of melanin in the fungal extract. The more the melanin is present, the higher is the particle size.

The microparticles produced were spherical and of regular shape, but with a slightly cracked surface. No significant difference was observed between chitosan and fungal extracts, other than for the mean size of the microparticles produced. Indeed, initial dry weight differed between fungal ethanol extracts and higher initial dry weights of the spray-dried sample were associated with larger mean microparticle size. This reflects a higher concentration of matter in the sprayed droplets.

Effect of Microencapsulated Matrices on the Growth of Enological Yeasts

The approach used here aimed to combine the antimicrobial properties of chitosan with the antioxidant properties of the fungal extract. No Brett Inside® is a commercial chitosan, obtained from the fungus *Aspergillus niger*, which is already on sale for limiting wine spoilage due to *Brettanomyces bruxellensis* [11]. This micro-organism is recognized as a major source of contamination in wine and as the principal cause of a "horse sweat" flavor [42]. *Brettanomyces bruxellensis* can develop in both musts and wine, throughout the entire winemaking process. As described by Taillandier et al., No Brett Inside® chitosan (NBI) seemed to be generally more effective than low molecular weight chitosan (LMW) in the winemaking context. NBI seemed to have less impact on the growth of *S. cerevisiae*, which is required for alcoholic fermentation, and a stronger impact on the growth of *B. bruxellensis*, the chief contaminant, than LMW (Fig. 2).

Conversely, the spray-drying technique has no impact on the slowing of *B. bruxellensis* development (Fig. 2b), whereas antioxidant supplementation of the chitosan matrix considerably decreased antimicrobial efficacy, at least for CE*3 and CE*6 supplementation. The addition of a too large quantity of antioxidant might, therefore, limit the antimicrobial efficacy of chitosan or strong presence of melanin in those matrices could limit the mode of action of chitosan.

Antioxidant Activity of Microencapsulated Matrices

Regarding the antioxidant capacity of the fungal extracts before microencapsulation, a relation can be found between the quantity of NGPs present in an extract and its antioxidant capacity. Indeed, an antioxidant capacity between 1.2- and 1.6-mM TEAC could be found per gram of NGPs present in the extract (not statistically different). The antioxidant capacity of NGPs have previously been described confirming this hypothesis [15, 16, 43].

Three types of microencapsulated matrix suspensions were tested: anhydrous ethanol (the solvent used to obtain the antioxidant fungal extract); acidified ethanol 12% (pH 3) to represent conditions equivalent to those found in wine; and acidified water (pH 3) to mimic grape must. Antioxidant activity was limited in anhydrous ethanol suspensions (Table 2) possibly due to the very low solubility of chitosan in organic solvents. In such conditions, the chitosan envelope would prevent the release of the antioxidant molecules into the medium, thereby limiting antioxidant activity. Alternatively, there may be an antagonistic effect between chitosan and the antioxidant molecule of the fungal extract in this type of solvent.

By contrast, in the suspensions simulating wine (12% ethanol) and must (acidified water), a synergistic effect on antioxidant properties was observed (Table 2).

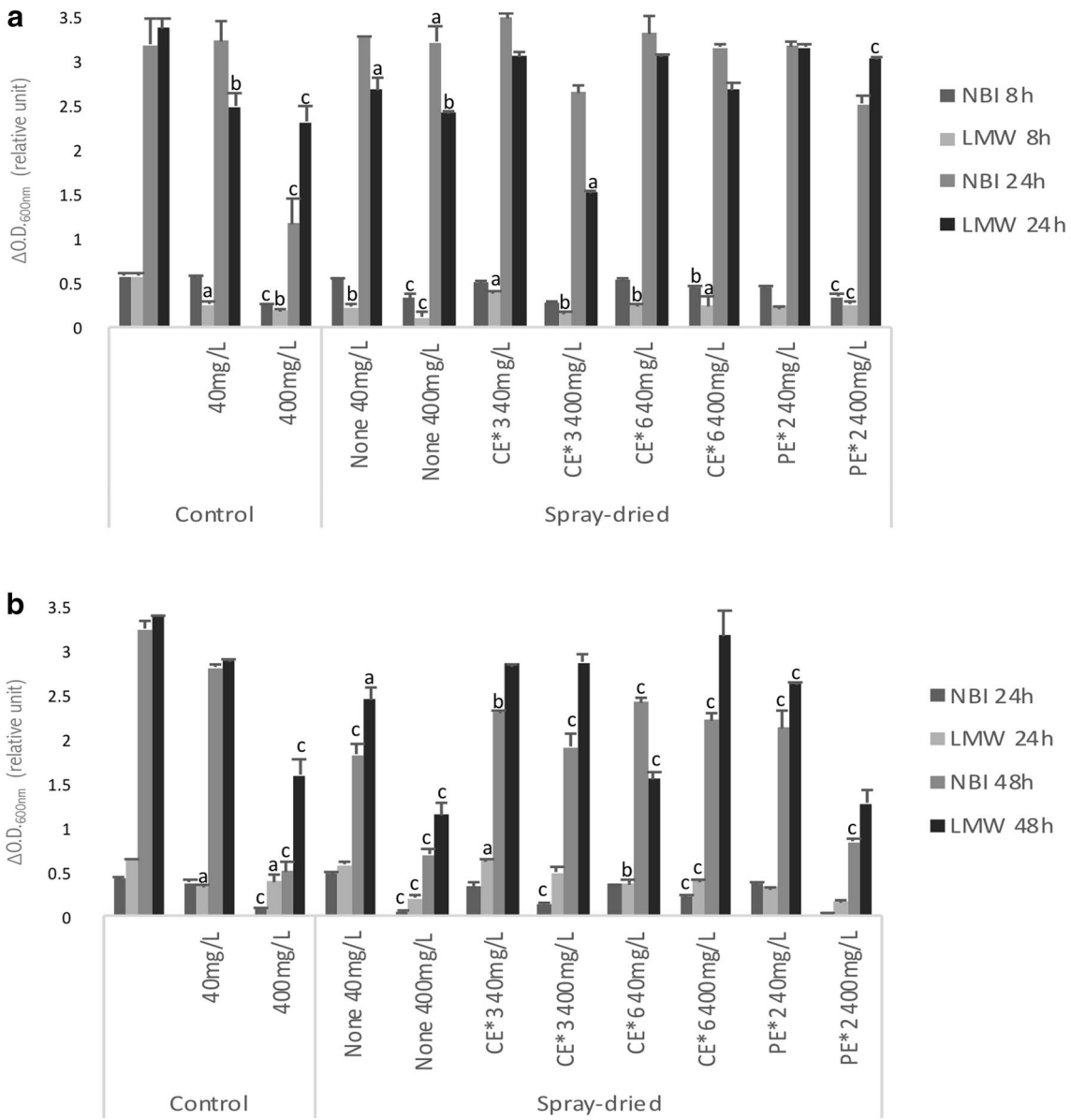

Fig. 2 Growth of *Saccharomyces cerevisiae* (**a**) and *Brettanomyces bruxellensis* (**b**) populations in the presence of different types of chitosan, as assessed by measuring ΔOD_{600nm}. *LMW* low molecular weight chitosan, *NBI* No Brett Inside® chitosan; *None* anhydrous ethanol control, *PE*2* SPE semi-purified fungal extract, *CE*3* fungal extract concentrated by rotary evaporation (3 V/V), *CE*6* fungal extract concentrated by rotary evaporation (6 V/V). Letters indicate values significantly different from the control (one-way ANOVA with Bonferroni post hoc correction; a: p-value < 0.05; b: p-value < 0.005; c: p-value < 0.001)

Discussion

This study shows that the supplementation of chitosan matrices, already commercially available, with an antioxidant extract, produced from a filamentous fungus isolated from a Mediterranean grapevine, could extend the use of this additive to the entire winemaking process as a sulfites alternative. As discussed in the introduction, sulfites are added during the winemaking process for two reasons: to limit the growth of undesirable microorganisms (antimicrobial properties) and to reduce the oxidation of grapes to preserve wine color by preventing juice browning (antioxidant properties). The greatest risk in must is enzymatic browning [44]. Alternatives to sulfites have already been proposed for preventing browning at this stage: salicylic acid during harvesting, glutathione or patatin, and a fining agent limiting browning [44–46]. In this study, an antioxidant extract produced from a fungus isolated from Mediterranean grapevines was selected as a natural product to supplement both LMW and NBI for use in the winemaking process. The two

Table 2 Antioxidant capacity of microencapsulated matrix suspensions at 3 g/L (dry weight)

	Ethanol 100%		Ethanol 12%		Acidified water	
	$TEAC_{est}$ (mM)	$TEAC_{exp}$ (mM)	$TEAC_{est}$ (mM)	$TEAC_{exp}$ (mM)	$TEAC_{est}$ (mM)	$TEAC_{exp}$ (mM)
Control						
CE*3		11.22 ± 0.53				
CE*6		18.66 ± 0.21				
PE*2		2.2 ± 0.08				
LMW		0.06 ± 0.001		0.22 ± 0.005		0.31 ± 0.025
NBI		N.D. ± 0		0.11 ± 0.002		0.19 ± 0.005
LMW						
CE*3	0.52	0.16^{d} ± 0.017	0.61	**1.15**^{b} ± 0.021	0.66	**1.56** ± 0.036
CE*6	0.61	0.38^{c} ± 0.016	0.68	**0.76**^{e} ± 0.005	0.72	**0.90** ± 0.018
PE*2	0.22	**0.44**^{c} ± 0.01	0.37	**1.09**^{b} ± 0.036	0.45	**1.4**^{a} ± 0.04
NBI						
CE*3	0.48	0.16^{d} ± 0.01	0.54	**1.26**^{a} ± 0.055	0.59	**1.4**^{a} ± 0.07
CE*6	0.59	0.40^{c} ± 0.009	0.63	**1.13**^{b} ± 0.056	0.67	**0.97**^{b} ± 0.018
PE*2	0.16	**0.34**^{c} ± 0.022	0.26	**0.83**^{e} ± 0.034	0.34	**0.94**^{b} ± 0.004

The mean of a triplicate analysis is indicated (SD < 0.07)

LMW low molecular weight chitosan, *NBI* No Brett Inside® chitosan, *PE*2* SPE semi-purified fungal extract, *CE*3* fungal extract concentrated by rotary evaporation (3 V/V), *CE*6* fungal extract concentrated by rotary evaporation (6 V/V), $TEAC_{est}$ estimated TEAC, $TEAC_{exp}$ experimental TEAC

Synergistic effect (SE) is indicated in bold; Antagonistic effect (AN) is underlined

Data with the same letter are not significantly different (two-way ANOVA with Tukey post hoc correction, p-value < 0.05)

natural active ingredients were combined by spray-drying microencapsulation to generate a powder that is easy to use and to store. The spray-drying technique seems to decrease the negative impact of chitosan on *S. cerevisiae* growth, with synergistic effects, increasing the antioxidant capacity of the mixture. Indeed, the growth of *S. cerevisiae* was significantly slowed in the presence of the commercial chitosan at the concentration recommended for limiting the growth of *B. bruxellensis* [11]. This slowing of growth has been reported to be due to a phenomenon of yeast absorption by the polymer [47]. This slower growth of *S. cerevisiae* may slow the alcoholic fermentation, prolonging the winemaking process, if chitosan is added to the must stage. It limits the use of the commercial NBI product for the treatment of wines before bottling. Interestingly, the effect of chitosan on *S. cerevisiae* growth seems to be reduced by spray-drying and/or antioxidant supplementation. This could allow a wider use of chitosan in winemaking processes, including its addition to the must before the initiation of alcoholic fermentation.

The experimental antioxidant capacity of most powders was two to three times higher than the estimated value for a combination of the antioxidant activities of chitosan and the chosen fungal extract [48]. The spray-drying of a combination of two antioxidant fungal extracts (*Suillus luteus* and *Coprinopsis atramentaria*) has already been shown to double the antioxidant activity over that expected (Ribeiro et al. [38]). A similar synergistic effect was observed for the chitosan/fungal extract combination, highlighting the potential value of this powder for the use in the winemaking process. PE*2 supplemented matrices had the strongest antioxidant properties, with a synergistic effect of 2.8 to 3.2 times the expected value. These results are consistent with those of Sansone and colleagues (2014), showing small particles size generally increases microparticle dissolution and the release of the encapsulated active ingredient (Sansone et al. [41]). Under laboratory conditions, in simple media, the antimicrobial and antioxidant properties were combined, revealing this powder to be a promising alternative to sulfites for use in winemaking. Under the conditions tested, the PE*2-supplemented matrix is a good candidate for use in wine applications. Indeed, the spray-drying technique limited the effects of chitosan on *S. cerevisiae* growth, whereas the effect on *B. bruxellensis* growth was similar to that of NBI used in the recommended conditions in wine (Control, 400 mg/L). The addition of the NBI PE*2 matrix to the must could therefore be considered in winemaking. However, for confirmation of this potential, scaling up of the fungal antioxidant production has to be shown in order to manufacture the process, and recent advances have been made on this step [49]. Then, both properties (antioxidant and antimicrobial) will need to be tested directly on a must, with evaluations of browning and microbial spoilage throughout the winemaking process and assessments of the

release of the active ingredients from the powder into a complex medium such as must or wine. Finally, innocuity of the product should be confirmed. However, previous studies have already shown beneficial effect on rats' health with a daily intake of NGPs [11, 50].

Author Contributions EC, VD, JR ,and FM designed the experiment. EC realizes the crude extract production, extract purification, antioxidant, and antimicrobial analyses. VD and IA realize microencapsulation, physicochemical properties of the obtained particles, and scanning electron microscopy. EC and VD wrote the manuscript; IA, JR ,and FM made a critical reading of the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest (financial, non financial).

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