International Journal of Food Microbiology 246 (2017) 1–4

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Effect of chitosan and SO_2 on viability of *Acetobacter* strains in wine

Maria José Valera, Florencia Sainz, Albert Mas, María Jesús Torija *

Biotecnologia Enológica, Dept. Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, C/ Marcel·lí Domingo s/n., 43007 Tarragona, Spain

ARTICLE INFO

Article history:
Received 18 November 2016
Received in revised form 10 January 2017
Accepted 31 January 2017
Available online 01 February 2017

Keywords:
Wine stabilization
Spoilage
Real-time PCR
Antimicrobial
Acetic acid bacteria

ABSTRACT

Wine spoilage is an important concern for winemakers to preserve the quality of their final product and avoid contamination throughout the production process. The use of sulphur dioxide (SO_2) is highly recommended to prevent wine spoilage due to its antimicrobial activity. However, SO_2 has a limited effect on the viability of acetic acid bacteria (AAB). Currently, the use of SO_2 alternatives is favoured in order to reduce the use of chemicals and improve stabilization in winemaking. Chitosan is a biopolymer that is approved by the European authorities and the International Organization of Vine and Wine to be used as a fining agent and antimicrobial in wines. However, its effectiveness in AAB prevention has not been studied.

Two strains of *Acetobacter*, adapted to high ethanol environments, were analysed in this study. Both chitosan and SO_2 effects were compared in artificially contaminated wines. Both molecules reduced the metabolic activity of both AAB strains. Although AAB populations were detected by culture independent techniques, their numbers were reduced with time, and their viability decreased following the application of both products, especially with chitosan.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

The control of wine stability throughout production and after bottling has been a major focus of the wine industry. A vinegary aroma or taste is a result of the presence of acetic acid produced by yeast, lactic acid bacteria and acetic acid bacteria (AAB). However, in the wine industry, the presence of AAB is the primary concern regarding high acetic acid levels. Environments with oxygen and temperatures between 20 and 30 °C are ideal for AAB growth, leading to increased volatile acidity and wine spoilage. In addition to producing a vinegar-like flavour, they produce undesirable characteristics such as bruised apple, sherry-like, nutty, wine glue or solvent aromas, as well as a reduction in fruity characters (Bartowsky et al., 2003). The presence of AAB is not readily detectable in wines because they are poorly recovered on plates (Torija et al., 2010; Trcek, 2005). Thus, the use of culture independent tools to identify AAB in wines has been recently implemented (González et al., 2006; Torija et al., 2010; Valera et al., 2015).

In wine cellars, SO_2 is used to prevent wine spoilage due to its antimicrobial properties. In addition, it is an antioxidant and limits the growth of indigenous microbiota. However, SO_2 is not completely effective at eliminating AAB in wine production; in fact, AAB can be detected in final products (Andorrà et al., 2008; González et al., 2005).

Winemakers tend to reduce SO_2 use due to adverse reactions in humans, including allergies to sulphites and sulphates (Bartowsky et al., 2003). Currently, wine consumers prefer less processed wines that maintain high quality. To meet this demand, development of new preservative agents or stabilization techniques are needed. Natural products from various organisms able to prevent microbial spoilage can be an alternative to the use of chemical products. According to these requirements, the chitosan molecule seems to be a good option for winemakers. Chitosan is a deacetylated derivative from the chitin shells of crustaceans. Its application in winemaking has been approved by the EU and OIV. In wines, chitosan has been shown to be effective as a fining and protein stabilizing agent (Chagas et al., 2012), preventing wine oxidation (Nunes et al., 2016) and as an antimicrobial against lactic acid bacteria, fungi and undesirable yeasts such as *Brettanomyces* during ageing (Ferreira et al., 2013; Nardi et al., 2014; Petrova et al., 2016) allowing the growth of *Saccharomyces* species (Elmacı et al., 2015). However, to our knowledge, chitosan has not been tested to control AAB spoilage in wines. The aim of this study was to compare the effect of chitosan and SO_2 in wines artificially contaminated with two high alcohol-resistant strains of AAB.

2. Materials and methods

2.1. Selection of AAB strains with high ethanol tolerance

Sixteen *Acetobacter* strains belonging to 4 different species (*Acetobacter pasteurianus*, *Acetobacter malorum*, *Acetobacter cerevisiae* and *Acetobacter tropicalis*) isolated by our research group from spontaneous microvinifications of healthy grapes from the Canary Islands were used (Valera et al., 2011).

* Corresponding author.
E-mail address: mjesus.torija@urv.cat (M.J. Torija).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.01.022
0168-1605/© 2017 Elsevier B.V. All rights reserved.

First, they were evaluated to select those best adapted to high ethanol concentrations. After a recovery step of 48 h at 28 °C in GY medium (5% glucose (w/v); 1% yeast extract (w/v)), the 16 strains were inoculated into synthetic wine (4 g/L tartaric acid; 0.134 g/L sodium acetate; 5 g/L glycerol; and 1.7 g/L yeast nitrogen base) with the pH adjusted to 3.5 and the ethanol concentration to 6% (v/v) to start their acclimation. Initially, the concentration of AAB was 10^8 cells/mL. The ethanol content of the synthetic wine was progressively increased for 21 days to achieve a final concentration of 11% (v/v), while maintaining the same amount of AAB for each ethanol addition step.

During this process, total titratable acidity was determined every 4 days by titration with 0.1 N NaOH, using phenolphthalein as an indicator (Ough and Amerine, 1987). This acidity is mainly due to the production of acetic acid. Moreover, AAB were quantified under the microscope using a Neubauer improved counting chamber (0.0025 mm^2 and 0.02 mm deep).

2.2. Inoculation of synthetic wines and storing conditions

The two strains adapted to the highest ethanol content, according Section 2.1, were inoculated in synthetic wine with 0.6% acetic acid (w/v) and 11% ethanol (v/v). Three experimental conditions were analysed in triplicate in 300 mL of wine distributed in 500 mL glass bottles: a) addition of SO_2 0.06 g/L; b) addition of chitosan (Bactiless, Lallemand Inc.) 0.2 g/L and c) control without treatment. These bottles were sampled at three different points after treatment: 2 weeks, 3 months and 6 months. Moreover, the wine immediately following inoculation was analysed as a baseline measurement (day 0).

Six 375 mL bottles were filled with 350 mL of synthetic wine inoculated with each of the selected strains. Half of these bottles were treated with 0.2 g/L of chitosan, and the remaining were untreated (controls). These bottles were then corked and stored at 25 °C for six months, after which they were opened and analysed.

2.3. Chemical analysis of wines

Sampling was done without shaking to avoid disturbing the sedimentation. For each sample, 10 mL of wine were centrifuged and analysed. Total titratable acidity was measured as described previously, and ethanol was measured using an enzymatic kit (Boehringer Mannheim, Germany).

2.4. Determination of total and viable population

Cell viability was determined using colony growth on GY solid medium (1% yeast extract, 5% glucose, 1.5% agar) supplemented with natamycin (100 mg/L) (Delvocid, DSM; Delft, The Netherlands) to suppress fungal growth. Then, 100 μL of undiluted or diluted wine, depending on the estimated population by microscopy, were plated. Plates were incubated for 48 h at 28 °C before counting.

Additionally, the viability of AAB was evaluated under a microscope using Live/Dead BacLight Kit (Molecular Probes, Eugene, OR, USA). Briefly, 10 mL of sample were centrifuged and pellets were stained with 1 μL of SYTO 9 dye and 1 μL of propidium iodide (PI) dye. After incubation in the dark for 20 min, each sample was washed with 500 μL of water to eliminate the excess dye; cells were then observed under epifluorescence microscopy.

Moreover, total AAB population was evaluated by real-time PCR (RT-PCR) using SYBR-Green fluorescent dye (González et al., 2006). Pellets from 10 mL of wine were used to extract genomic DNA according to the method described by Ausubel et al. (1992) using CTAB (cetyltrimethylammonium bromide). Calibration curves were constructed using a ten-fold dilution series of DNA extracted from 10^8 cells/mL of pure cultures of both selected strains. The reactions were performed with primers AQ1F and AQ2R (González et al., 2006) in a concentration of 600 nM for each primer and 5 μL of DNA solution, 12.5 μL SYBR-Green universal PCR master mix (Applied Biosystems, Foster City, CA, USA) yielding a final reaction volume of 25 μL, adjusted with MilliQ water. Amplifications were carried out on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Initially, the reaction was incubated at 50 °C for 2 min and then 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. CT values were automatically determined by the instrument, and NTC was considered PCR negative controls.

The variances of the results were analysed statistically using one-factor ANOVA and Student's *t*-test with a significance level of 5%.

3. Results

3.1. AAB strains selection

Throughout the selection process, ethanol concentration was progressively increased to adapt strains to the final synthetic wine characteristics. Among the 16 strains of *Acetobacter* tested, two strains, Am17 and Ap6, belonging to the species *A. malorum* and *A. pasteurianus* respectively, were selected because they had the highest ethanol tolerance and production of acetic acid.

Then, both strains were inoculated into synthetic wines containing a final total titratable acidity of 0.6 g of acetic acid/100 mL and 11% (v/v) ethanol. The initial population of each strain was determined by RT-PCR, which revealed 1.76×10^6 cells/mL for Ap6 and 5.62×10^5 cells/mL for Am17. However, when these samples were plated, no colonies were recovered from the wines inoculated with Am17, even undiluted. Instead, using the Live/Dead viability kit, cells were stained green, indicating that they were viable although not culturable. In the case of Ap6, similar counts were obtained in all the methods used to estimate the population, showing that the cells were viable and culturable.

3.2. Chemical parameters

Analysis 2 weeks after inoculation indicated that wines exhibited the same chemical characteristics as day 0 (Table 1). However, after 3 months, the acidity in control wines inoculated with Ap6 increased up to 1.1 g/100 mL acetic acid, but in wines treated with SO_2 the increase was lighter (0.7 g/100 mL acetic acid) and with chitosan there was no change in acetic acid levels. After 6 months, all the samples contaminated with Ap6 increased their acidity, especially the controls (yielding a mean of 2 g/100 mL of acetic acid) and those with the addition of chitosan or SO_2, which increased from 0.6 and 0.7 to 0.8 g/100 mL of acetic acid. The acidity increase in control compared to chitosan treatment were significantly different after 3 and 6 months. Total acidity in the samples inoculated with Am17 did not change by 6 months.

On the other hand, after 3 months, in the control and SO_2 treated wines, the concentration of ethanol decreased to 10% (v/v), while it was maintained at 11% (v/v) with chitosan treatment. These results were similar after 6 months for both strains and treatments, except in control Ap6, where a mean value of 9.5% (v/v) ethanol was observed, consistent with the increased acidity. Curiously, the wines inoculated with Am17 displayed significant differences in ethanol content between the treatments after 6 months.

3.3. AAB viability

In plated samples after 2 weeks and 3 months, colonies were only recovered from control wines inoculated with Ap6 (3.5×10^5 cfu/mL). However, after 6 months, colonies were obtained from one replicate of the Ap6 and Am17 controls (9×10^5 and 2×10^4 cfu/mL, respectively).

On the other hand, viability measurements based on the Live/Dead viability kit revealed differences between the three conditions assayed. After 15 days of inoculation, most cells were viable in control and SO_2 treated wines. Conversely, there were differences in chitosan treated wines; all of the chitosan treated Ap6 cells were damaged, while

Table 1
Results of chemical parameters analysis for all treatments and controls immediately after inoculation and in each sample point.

			0 days		2 weeks		3 months		6 months	
Inoculated strain	Bottle volume	Treatment	Acidity[a]	Ethanol[b]	Acidity	Ethanol	Acidity	Ethanol	Acidity	Ethanol
A. pasteurianus Ap6	500 mL	Control	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	11.0 ± 0.1	1.1 ± 0.3	10.3 ± 0.4	2.0 ± 0.9	9.5 ± 0.8
		SO_2	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	11.0 ± 0.1	0.7 ± 0.2	10.3 ± 0.3	0.8 ± 0.2	10.1 ± 0.3
		Chitosan	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	11.0 ± 0.2	0.6 ± 0.2	11.0 ± 0.7	0.8 ± 0.2	10.1 ± 0.4
	375 mL	Control	0.6 ± 0.2	11.0 ± 0.1	nd	nd	nd	nd	0.8 ± 0.2	10.2 ± 0.2
		Chitosan	0.6 ± 0.2	11.0 ± 0.1	nd	nd	nd	nd	0.5 ± 0.2	10.5 ± 0.3
A. malorum Am17	500 mL	Control	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	10.1 ± 0.4	0.6 ± 0.2	10.5 ± 0.1
		SO_2	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	10.4 ± 0.5	0.6 ± 0.2	10.2 ± 0.1
		Chitosan	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	11.0 ± 0.1	0.6 ± 0.2	11.5 ± 0.5	0.6 ± 0.2	10.8 ± 0.1
	375 mL	Control	0.6 ± 0.2	11.0 ± 0.1	nd	nd	nd	nd	0.6 ± 0.2	10.6 ± 0.3
		Chitosan	0.6 ± 0.2	11.0 ± 0.1	nd	nd	nd	nd	0.4 ± 0.2	11.2 ± 0.3

[a] Total acidity is expressed as g of acetic acid per 100 mL.
[b] Ethanol content is expressed as % (v/v).

wines inoculated with Am17 showed similar proportions of live and damaged cells. The amount of cells in suspension decreased after 3 months; in fact, no cells were visualized. Regardless of the treatment, wines after 6 months did not present enough cells in suspension to observe under the microscope, even after concentration of 100 mL.

3.4. RT-PCR analysis

The bacteria in suspension were quantified using RT-PCR, with primers specifically designed for AAB, using conserved regions in 16S rRNA that are common for all genera of this bacterial group. In control wines inoculated with Ap6, the population 2 weeks after inoculation decreased by one log unit (Fig. 1a). Interestingly, wines treated with SO_2 displayed a significantly higher amount of AAB relative to the control. Chitosan treated wines exhibited no significant differences between control and SO_2 treatment. The population of AAB in chitosan and SO_2 treated wines decreased with time. After 3 months, the AAB levels with chitosan treatment were significantly different than control and SO_2 treated wine. Populations of AAB in treated wines decreased after 3 months but were maintained in the control. After 6 months, the treated samples maintained the same AAB populations while a significant increase (2–3 orders of magnitude) was observed in control.

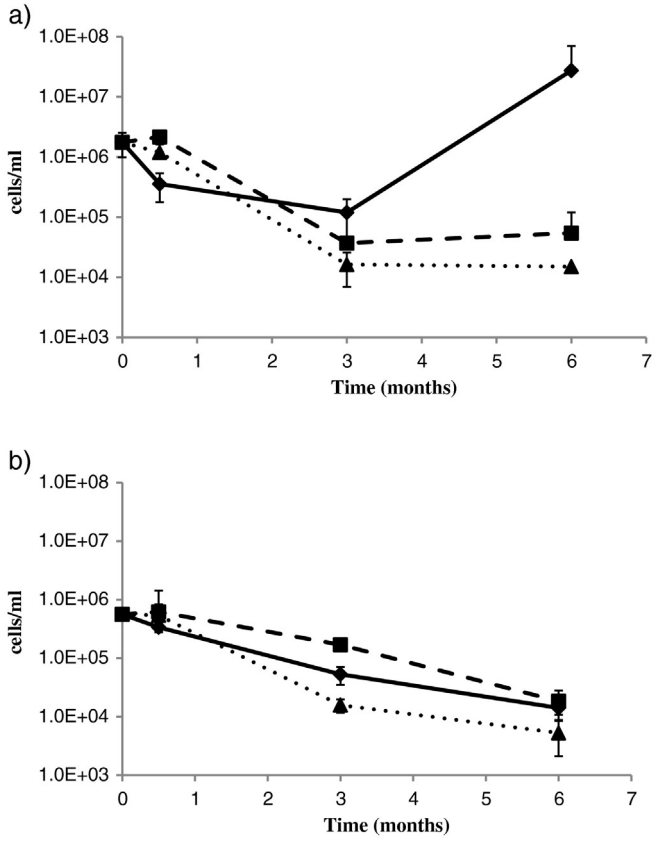

Fig. 1. Evolution of AAB population quantified by RT-PCR. Dotted lines (·····) represent chitosan treated samples and dashed lines (- - -) SO_2 treated ones. Solid lines (—) represent untreated controls. Population dynamics: a) *A. pasteurianus* strain Ap6 and b) *A. malorum* strain Am17. Bars represent standard error.

RT-PCR revealed that the Am17 control wines contained similar populations two weeks after inoculation to those seen with Ap6; in addition, AAB populations in SO_2 and chitosan treated wines were slightly higher (Fig. 1b). Nevertheless, after 3 months, the difference between the treatments and the control was statistically significant and there was a one log unit lower population of AAB in chitosan than in SO_2 treated wines. The population in the control wine was between chitosan and SO_2. Six months after inoculation, the control and SO_2 treated wines displayed similar populations estimated by RT-PCR. These quantification results from the control and SO_2 were statistically higher compared with the chitosan samples.

3.5. Effect of chitosan on corked bottles

Wines bottled with and without the addition of chitosan were analysed 6 months after corking. The acetic acid concentration in Ap6 inoculated, chitosan treated wine was 0.5 g/100 mL compared to 0.8 g/100 mL in the control (Table 1). In Am17 inoculated wine, the acetic acid concentration was 0.4 g/100 mL in chitosan treated and 0.6 g/100 mL in the control.

The ethanol concentration with chitosan treatment was 10.5% (v/v) and 11% (v/v) for wines inoculated with Ap6 and Am17, respectively. In the control, the ethanol amount was slightly lower for both strains, although the difference was statistically significant only for Am17.

All of the controls yielded colonies on GY medium with a similar average population of 30 cfu/mL and 33 cfu/mL for Ap6 and Am17, respectively. However, the chitosan treated samples yielded no colonies. Using the Live/Dead viability kit, it was possible to detect under the microscope that cells from both strains in the control were alive and that no cells were visible in the preparations from chitosan treated wines, even when they were 100 times more concentrated before dyeing them.

Regarding the results obtained with RT-PCR, the Ap6 strain presented significant differences in AAB populations of the control and chitosan treated wines. However, the Am17 strain did not present significant differences.

4. Discussion

Although AAB from culture collections are stored in optimal conditions, most strains lose their ability to survive under adverse conditions,

even when isolated from extreme environments such as wines or vinegars (Sokollek et al., 1998). Due to the extreme conditions in wines that are suboptimal for the direct growth of stored AAB strains, it is important that strains with specific adaptations are selected. Key characteristics of strains adapted for growth in wine include the ability to thrive in environments with high ethanol and lack of nutrients.

The 16 strains tested from the culture collection belonged to *Acetobacter*, an AAB genus commonly found in wines (Joyeux et al., 1984), including the two selected species, *A. pasteurianus* and *A. malorum*. The former species has been reported as highly resistant to ethanol and SO_2 (Du Toit and Lambrechts, 2002), while the latter has been identified in spontaneous fermentation of grape must (Valera et al., 2011). Some of the strains previously identified as other *Acetobacter* species may actually belong to *A. malorum*. Therefore, the adaptation of Ap6 and Am17 to thrive in wine conditions was not surprising.

After the selection process and adaptation of the selected strains, Ap6 but not Am17 was able to grow on plates, even though viability appeared to be equivalent. This is not surprising based on previous attempts to culture AAB isolated from wine with common culture media (Millet and Lonvaud-Funel, 2000; Torija et al., 2010). Moreover, viable populations in suspension were inconsistent at various time points in the controls. Ap6 was able to grow at day 0 and 3 months, but not after 2 weeks and 6 months. These inconsistencies could be due to the difficulties of recovering AAB directly from wines and culturing on GY medium, which represents an abrupt change in conditions that the bacteria may not be adapted to. Nevertheless, the viability was also analysed under the microscope. The use of special dyes that mark viable cells has been used in the assessment of AAB. This technique revealed differences of up to four orders of magnitude between viable bacteria counts by microscopy and culturable populations (Baena-Ruano et al., 2006; Mesa et al., 2003).

After 2 weeks, based on Live/Dead dyes, a high concentration of damaged cells was evident in wines treated with chitosan. However, in the control, fewer cells were observed, but they were viable. The amount of cells in suspension decreased with time in Am17, regardless of treatment. By 3 months, the cells in suspension appeared viable, although at low concentrations due to the increase of acetic acid and the lack of aeration. A lack of detectable damaged cells could be due to their sedimentation to the bottom of the bottle. AAB need high aeration to survive, so they tend to reside on the surface of liquids (Valera et al., 2015).

The inconsistencies between RT-PCR data and the estimation of viability by microscopy after 2 weeks when populations are higher could potentially be explained by PCR amplification of DNA in suspension, which does not necessarily represent viable cells. However, by 3 months, the number of cells in suspension was too low to obtain reliable viability approximations based on microscopy. The RT-PCR values progressively decreased with the time, potentially indicating that the cells precipitated after death.

The 500 mL bottles were exposed to more oxygen because of their air chamber and the frequency of sampling, which was higher than corked bottles. In the wine industry, although AAB can be isolated from wines in semi-anaerobic conditions, they do not retain their ability to grow. In strictly aerobic microorganisms such as AAB, oxygen is required for growth. After bottling, lack of air and oxygen prevents spoilage; however, excessive aeration prior to bottling or the incorrect storage conditions can increase the occurrence of AAB (Bartowsky et al., 2003; Millet and Lonvaud-Funel, 2000). This explains why corked bottles maintained similar AAB populations after 6 months, while AAB populations increased in untreated wines inoculated with Ap6 in 500 mL bottles.

Chitosan is a biopolymer able to form complexes with iron and tartrate promoting the decrease of iron availability, preventing the growth of microorganisms (Nunes et al., 2016). The use of chitosan as wine preservative has been proven to avoid chemical alterations and also the appearance of spoiler yeasts and fungi (Chagas et al., 2012; Nunes et al., 2016; Nardi et al., 2014; Petrova et al., 2016). Complementarily, in the present study, the treatment with chitosan was found to be effective in reducing the activity of AAB in wine. This reduction is more evident immediately following application, as well as in strains with more activity. This effectiveness is detected by culture-dependent and -independent assessments; especially remarkable is effect on acetic acid production and ethanol consumption. Also, in the case of lactic acid bacteria, an immediate effect of chitosan was observed by the arrest of their growth (Elmacı et al., 2015). Taken together, these data indicate that chitosan reduces AAB population quantity and metabolic activity. Therefore, chitosan appears to be at least as effective as SO_2 in wine preservation.

Acknowledgements

The authors thank Lallemand Inc. for providing the chitosan used for the realization of these experiments.

References

Andorrà, I., Landi, S., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B., 2008. Effect of oenological practices on microbial populations using culture-independent techniques. Food Microbiol. 25, 849–856.
Ausubel, F.M., Brent, R., Kingston, R., Moore, D.D., Seidman, J.G., Smith, J.A., Struh, K., 1992. Short protocols. Molecular Biology. John Wiley.
Baena-Ruano, S., Jiménez-Ot, C., Santos-Dueñas, I.M., Cantero-Moreno, D., Barja, F., García-García, I., 2006. Rapid method for total, viable and non-viable acetic acid bacteria determination during acetification process. Process Biochem. 41, 1160–1164.
Bartowsky, E.J., Xia, D., Gibson, R.L., Fleet, G.H., Henschke, P.A., 2003. Spoilage of bottled red wine by acetic acid bacteria. Lett. Appl. Microbiol. 36, 307–314.
Chagas, R., Monteiro, S., Ferreira, R.B., 2012. Assessment of potential side effects of common fining agents used for white wine protein stabilization. Am. J. Enol Vitic., ajev-2012
Du Toit, W.J., Lambrechts, M.G., 2002. The enumeration and identification of acetic acid bacteria from South African red wine fermentations. Int. J. Food Microbiol. 74, 57–64.
Elmacı, S.B., Gülgör, G., Tokatlı, M., Erten, H., İşci, A., Özçelik, F., 2015. Effectiveness of chitosan against wine-related microorganisms. Anton Leeuw. Int. J. G. 107, 675–686.
Ferreira, D., Moreira, D., Costa, E.M., Silva, S., Pintado, M.M., Couto, J.A., 2013. The antimicrobial action of chitosan against the wine spoilage yeast *Brettanomyces/Dekkera*. J. Chitin Chitosan Sci. 1, 240–245.
González, A., Hierro, N., Poblet, M., Mas, A., Guillamón, J.M., 2005. Application of molecular methods to demonstrate species and strain evolution of acetic acid bacteria population during wine production. Int. J. Food Microbiol. 102, 295–304.
González, A., Hierro, N., Poblet, M., Mas, A., Guillamón, J.M., 2006. Enumeration and detection of acetic acid bacteria by real-time PCR and nested PCR. FEMS Microbiol. Lett. 254, 123–128.
Joyeux, A., Lafon-Lafourcade, S., Ribéreau-Gayon, P., 1984. Evolution of acetic acid bacteria during fermentation and storage of wine. Appl. Environ. Microbiol. 48, 153–156.
Mesa, M.M., Macías, M., Cantero, D., Barja, F., 2003. Use of the direct epifluorescent filter technique for the enumeration of viable and total acetic acid bacteria from vinegar fermentation. J. Fluoresc. 13, 261–265.
Millet, V., Lonvaud-Funel, A., 2000. The viable but non-culturable state of wine micro-organisms during storage. Lett. Appl. Microbiol. 30, 136–141.
Nardi, T., Vagnoli, P., Minacci, A., Gautier, S., Sieczkowski, N., 2014. Evaluating the impact of a fungal-origin chitosan preparation on *Brettanomyces bruxellensis* in the context of wine aging. Wine Studies 3 (4574), 13–15.
Nunes, C., Maricato, É., Cunha, Â., Rocha, M.A.M., Santos, S., Ferreira, P., Silva, M.A., Rodrigues, A., Amado, O., Coimbra, J., Silva, D., Moreira, A., Mendo, S., Lopes da Silva, J.A., Pereira, E., Sílvia, M., Rocha, S.M., Coimbra, M.D., 2016. Chitosan–genipin film, a sustainable methodology for wine preservation. Green Chem. 18, 5331–5341.
Ough, C.S., Amerine, M.A., 1987. Methods for Analysis of Must and Wines. Wiley-Interscience Publication, California.
Petrova, B., Cartwright, Z.M., Edwards, C.G., 2016. Effectiveness of chitosan preparations against *Brettanomyces bruxellensis* grown in culture media and red wines. OENO One 50, 49–56.
Sokollek, S.J., Hertel, C., Hammes, W.P., 1998. Cultivation and preservation of vinegar bacteria. J. Biotechnol. 60, 195–206.
Torija, M.J., Mateo, E., Guillamón, J.M., Mas, A., 2010. Identification and quantification of acetic acid bacteria in wine and vinegar by TaqMan-MGB probes. Food Microbiol. 27, 257–265.
Trcek, J., 2005. Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S–23S rDNA internal transcribed spacer region and of the PQQ-dependent alcohol dehydrogenase gene. Syst. Appl. Microbiol. 28, 735–745.
Valera, M.J., Laich, F., González, S.S., Torija, M.J., Mateo, E., Mas, A., 2011. Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands. Int. J. Food Microbiol. 151, 105–112.
Valera, M.J., Torija, M.J., Mas, A., Mateo, E., 2015. Acetic acid bacteria from biofilm of strawberry vinegar visualized by microscopy and detected by complementing culture-dependent and culture-independent techniques. Food Microbiol. 46, 452–462.