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1 # Effect of kaolin silver complex on the control of populations of
2 # *Brettanomyces* and acetic acid bacteria in wine.

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Abstract

The aim of this work was to study the effects of kaolin silver complex (KAgC) on the control of populations of *Brettanomyces* and acetic acid bacteria in winemaking.

We show that the KAgC in wine at doses of 1 g/L provides effective control against the development of *Brettanomyces* and acetic acid bacteria. In the wines artificially contaminated with an initial population of 10^4 CFU/mL of *B. bruxellensis*, it was possible to reduce almost 3 log on the third day of treatment with KAgC and only residual populations of the contaminating yeast (24 CFU/mL) remained after 24 days of contact with the additive. Irrespective of the initial population of *Brettanomyces*, wines with KAgC showed lower concentrations of acetic acid and 4-ethyl-phenol than wines without KAgC. The population of acetic bacteria inoculated in wine at concentrations of 10^2 and 10^4 CFU/mL was reduced to negligible levels after 72 hours of treatment with KAgC.

The antimicrobial effect of KAgC in a wine naturally contaminated with *Brettanomyces bruxellensis* was similar to that which occurs in the treatment with chitosan, decreasing at 10 days and in both cases by 2 log with regard to the initial contaminating population. The effect of the treatment with KAgC also reduced the population of acetic bacteria by 2 log the initial level of population. Silver concentration of KAgC added in finished wines was below the legal limits.

Keywords: Acetic acid bacteria, *Brettanomyces*, Chitosan, Kaolin-Silver, Wine

Introduction

Wine quality is greatly influenced by the microorganisms which occur throughout the winemaking process. It has been shown that yeasts belonging to the species *Dekkera bruxellensis*, or its anamorph *Brettanomyces bruxellensis,* have the capacity of spoiling wines by producing ethyl phenols (Loureiro and Malfeito-Ferreira 2006), which are the compounds responsible for the off-flavors described as animal odors, farmyards, horse sweat, medicine and animal leather (Chatonnet *et* al. 1995). For many years, barrel aging has been considered a source of spoilage. However, better surveys of the yeast population and spoilage has clearly shown that the problem could occur even during alcoholic fermentation in stainless steel tanks and also during aging process.

Brettanomyces associated problems have seemingly become more prominent in recent years as a consequence of lower sulfur dioxide (SO_2) usage due to pressing consumer demands, the increase of pH that lowers the SO_2 efficiency and the favorable conditions during aging in barrels (Du Toit et al. 2005, Renouf et al. 2006). Various authors have concluded that controlling the growth of *Brettanomyces* is the most important challenge for modern winemaking (Wedral et al. 2010).

Moreover, acetic acid bacteria (AAB) play a negative role in wine, being one of the main reasons for wine spoilage (Drysdale and Fleet 1988) because of an undesirable production of acetic acid, acetaldehyde, ethyl acetate and dihydroxyacetone (Sponholz and Dittrich 1984). Till now, sulfur dioxide addition has been the main way to inactivate spoilage microorganisms. Nevertheless, there is a worldwide trend to reduce sulfur dioxide levels in wine due to several factors such as increasing health concerns, consumer preferences, possible organoleptic alterations in the final product and potential legislation on preservatives (García-Ruiz et al. 2013). For this reason, there is particular interest within the scientific community in the development of alternatives to the traditional use of sulfur dioxide in winemaking (Izquierdo-Cañas et al. 2012, González-Arenzana et al. 2015, González-Arenzana et al. 2016).

Among these alternatives, chitosan has received considerable attention due to the approval of its use in treatments for wine by the International Organization of Vine and Wine at the OIV Resolution 338A-2009 (OIV, 2015) notably for the *Brettanomyces* control. There are several studies dealing with the application of chitosan in various food products (Giatrakou et al. 2010, Huang et al. 2012, Giner et al. 2012). The effectiveness of chitosan against *Brettanomyces bruxellensis* has been examined in mixed culture fermentations (Gómez-Rivas et al. 2004), in vitro conditions (Elmaci et al., 2015, Petrova et al. 2016), in a wine-model synthetic medium (Taillander et al. 2014), and in real vinifications and commercially produced wines (Blateyron-Pic et al. 2012, Ferreira et al. 2013, Petrova et al. 2016). However, as Petrova et al. (2016) concluded, wines treated with chitosan were not completely stable after treatment, as populations eventually increased. Furthermore, chitosan can negatively affect some physicochemical characteristics of wine (Ferreira et al. 2013).

An alternative to the addition of SO_2, from the point of view of its antimicrobial action, is the use of silver. Silver has been used for its antimicrobial properties since ancient times and

recent studies have shown that silver nanomaterials are antimicrobial towards a broad spectrum of Gram-positive and Gram-negative bacteria and also exert some antifungal and antiviral activities (Rathnayake et al. 2012, García-Ruíz et al. 2015). Despite the great interest in the applications on these materials in the field of enology, so far studies on the use of silver as an antimicrobial in winemaking have been very scarce (Monge et al. 2016).

This study shows the results of two trials that examine the effects of kaolin silver complex (KAgC) (Enosan Micro. Laboratorios Enosan, S.L., Zaragoza, Spain, www.laboratoriosenosan.com) on the control of populations of *Brettanomyces* and acetic acid bacteria in winemaking and provides comparison with chitosan on *Brettanomyces* control. The effect of KAgC treatment on the metabolites of a *Brettanomyces* and AAB contamination (acetic acid and volatile phenols) is also shown.

Material and methods

KAgC (Kaolin Silver Complex)

KAgC is produced under patent (PCT/ES2015/070532). It is a grey powder with particle size of around 30 nm and it is insoluble in ethanol and water, composed of an inorganic inert material (kaolin), used as support, on whose surface silver nanoparticles (<10 nm) are deposited (colloidal silver). KAgC was supplied in permeable bags that contained 1 g of KAgC.

Initial wines

In trial 1 a red wine was used (**Table 1**, Wine 1) which has been produced without the addition of sulfur dioxide, but with a natural presence as a secondary metabolite of total SO_2 (≤ 4 mg/L). The wine was fined with egg white and filtered through 0.22 microns in order to eliminate any naturally occurring contaminating microorganism in the wine. Prior to commencing the experiment, a check that the wine is sterile was performed by filtering 100 mL of wine through a 0.22 micron pore membrane and incubating it on a Sabouraud agar medium. After 48 h of incubation at 28 °C growth was null. The red wine was distributed in 36 aliquots of 1 L in previously sterilized glass bottles, with a magnetic stirrer in its interior to produce gentle agitation.

In the second trial a naturally contaminated wine (**Table 1**, Wine 2) with a population of *Brettanomyces* yeasts of 1.0E+04 CFU/mL and a population of AAB of 1.10E+05 CFU/mL was used. According to the results of the microbiological analysis, this wine did not contain lactic acid bacteria. The wine had a moderately high acetic acid content related to the presence of AAB. Also, the initial wine had high ethyl phenol and ethyl guayacol content related to the presence of the *Brettanomyces* yeasts.

Strains used to contaminate the wine from Trial 1

Brettanomyces

The initial culture of *Brettanomyces bruxellensis* consisted of a mixture of four strains isolated from a naturally contaminated wine that contained 742 µg/L of 4-ethyl-phenol. These four strains were identified at specie level as *Brettanomyces bruxellensis* by molecular techniques. The amplification of the internal transcribed spacers (ITS1 and ITS2) of the rRNA 5.8S was used. DNA from each culture was isolated and PCR amplification was carried out following conditions described by Guillamón et al. 1998 and Esteve-Zarzoso et al. 1999. PCR products were electrophoresed on 1.4 % agarose (Roche Diagnostics, Spain), stained with ethidium bromide and photographed (**Figure 1**). Results of the amplicon size were compared with those described in the bibliography (Guillamón et al. 1998 and Esteve-Zarzoso et al. 1999) and identified as *Brettanomyces bruxellensis*.

To obtain enough population to be inoculated in the Wine 1, these 4 strains were initially multiplied in YPD media. The experiment was carried out with a culture of *Brettanomyce*s with an initial concentration of 1.7E+07 CFU/mL. Different volumes of this culture were added to Wine 1 to get a population of 1.0E+02 CFU /mL, 1.0E+04 CFU/mL and 1.0E+06 CFU/mL respectively.

AAB (acetic acid bacteria)

To contaminate the Wine 1, two different species of acetic acid bacteria, *Gluconobacter oxydans* (Colección Española de Cultivos Tipo CECT 360) and *Acetobacter aceti* (CECT 298) were used. Bacteria were initially multiplied on Mannitol media (0,5 % yeast extract, 0,3 % peptone, 2,5 % Mannitol) to obtain sufficient population to be subsequently inoculated in wine to reach concentrations of 1.0E+02 CFU/mL, 1.0E+04 CFU/mL and 1.0E+06 CFU/mL, respectively.

Experiments and treatments

In trial 1, two experimental series (*Brettanomyces* and AAB) were made. Each experimental series consisted of bottles containing wine 1: 3 bottles were inoculated with *Brettanomyces* and 3 with AAB cultures to obtain 1.0E+02 CFU/mL, the same was done with 1.0E+04 CFU/mL and with 1.0E+06 CFU/mL concentrations. One bag of KAgC was added to 3 bottles of each series (triplicates), so the dose of treatment was 1 g/L and in each experimental series (Brett or AAB) 3 bottles without KAgC were used as a control.

Bottles were gently stirred (100 rpm) in order to put the bag that contained KAgC in contact with the whole volume of the wine. Samplings were taken at different contact times and plate count analysis, and acetic acid and 4-ethyl-phenol content were evaluated. The duration of the experiment of *Brettanomyces* inactivation was 24 days and 3 days for AAB.

In trial 2, three batches of 3 bottles each were prepared using Wine 2 (**Table 1**). One batch was used as a control; 1 g/L of KAgC was added to the bottles of the second batch and 7 g/HL of chitosan (NoBrettInside®, Lallemand, Montreal, Canada) to the last one. Each bottle was stirred daily and *Brettanomyces* and AAB populations were measured at day 10 of the treatment.

Microbiological counts

Enumeration by counts in plates

In Trial 1, the *Brettanomyces* population was controlled at day 0, just after being inoculated, and at 3, 10, 17 and 24 days after the treatment. Serial dilutions (from 10^{-1} to 10^{-6}) in sterile saline solution were plated onto Sabouraud-chloramphenicol agar plates (Cultimed, Panreac, Barcelona, Spain). Plates were incubated under aerobic conditions at 28ºC for 10 days. After this time colonies were counted and the results were expressed as colony forming units (CFU) per milliliter of wine.

The AAB population was counted in each bottle at day 0, just after being inoculated in the wine, and at 1, 2 and 3 days after starting the treatment with KAgC. 0.1 mL of the sample was taken and serial dilutions (from 10^{-1} to 10^{-6}) in a sterile saline solution were spread onto plates of GYC medium (5% glucose, 1% yeast extract, 0.5% calcic carbonate, 2% agar) to which 50 mg/L nystatin (Sigma-Aldrich) was added. The plates were incubated under aerobic

conditions at 30ºC for 5 days. Counts were expressed as colony forming units (CFU) per milliliter of wine.

In Trial 2, cell population was evaluated by qPCR based on Scorpions (Whitcobe *et al*. 1999; Umiker *et al*., 2013). qPCR detection was performed using the Scorpions Wine Spoilage Systems module (ETS Laboratories, St. Helena, CA).

After mixing, a 1.5 mL sample was removed and centrifuged (9,000 x g). The pellet was suspended in 1x wash buffer from the lysis module (LYR-50-01) and centrifuged. The pellet was then suspended in 15 mL of 1x wash buffer prior to transfer to a 15 mL centrifuge tube for recentrifugation. Cell lysis was accomplished by suspending cell pellets in 200 µL of 1x lysis reagent (LYR-50-01). Pellets were then incubated at 37ºC for 30 min, mixed and incubated for an additional 30 min adding 20 µL Proteinase K with 200 µL of PBS and buffer AL 200 uL (DX Reagent Qiagen Pack for QIAxtractor #950107, Qiagen, Inc., Valencia, CA) to the suspension and incubated for 30 min at 55ºC, mixed and incubated for an additional 30 min. Cell debris was removed by centrifugation (15,000 x g for 6 min) before removal of 420 µL supernatant used for DNA extraction and purification using the QIAxtractor and DX reagent pack according to the manufacturer's instructions.

For *Brettanomyces* purified nucleic acid (5 µL) was combined with 20 µL Scorpions Yeast Assay Multiplex Mastermix and 5 µL Scorpions Reagent (YDR1-50-01) along with 15 µL Taq Polymerase Mastermix containing dNTPs, $MgCl_2$ and supplied buffer. Some way for acetic acid bacteria using purified nucleic acid (5 µL) was combined with 20 µL Scorpions Bacteria I Assay Multiplex Mastermix and 5 µL Scorpions Reagent. Amplification and detection of DNA was conducted with a Q-Gene thermocycler (Qiagen, Inc.). Quantification of samples and efficacy of the assay was determined using standard curves generated by isolating DNA from serial dilutions (10^6–10^1) of a *Brettanomyces* and AAB respectably cultures grown in wine. The Scorpions Yeast and Bacteria Multiplex assay contain an internal control reaction consisting of primers and a probe to amplify target DNA spiked into the mastermix. Signal strength of the internal control reaction is monitored to avoid false negatives due to the presence of PCR inhibitors.

Positive controls, samples with a known population of *Brettanomyces* and acetic bacteria in wine, were lysed, extracted and amplified along with samples being analyzed. A non template control consisting of 20 µL yeast Scorpions Assay Multiplex Mastermix and 5 µL of

molecular biology grade ddH_2O was also conducted. Populations of *Brettanomyces* were calculated by the analysis software provided with the Q-Gene thermocycler.

Determination of acetic acid and volatile phenols

Evolution of acetic acid was analyzed in each sampling in Trial 1. Acetic acid was determined in a Lisa 200 multi-parametric analyzer (Hycel diagnostics, TDI Tecnología Difusión Ibérica, S.L., Spain) by enzymatic methods in accordance with Commission Regulation (EC 2676/1990, E.E.C., 1990) and the International Organization of Vine and Wine (OIV, 2016).

Ethylphenol in wines was analyzed by gas chromatography (Chatonnet et al. 1995) at the end of Trial 1 (day 24) in the treatment of *Brettanomyces* inactivation with KAgC. Ten mL of wine were extracted three times with successively 5 ml, 2 mL and 2 mL of dichloromethane. The combined organic extracts were slowly concentrated to 1 ml at room temperature by evaporation under nitrogen gas flow. Gas chromatography was performed with a HP5890 series II instrument by injecting 1 μL of the concentrate extract by means of a splitless injector (splitless time: 30 s; split ratio: 1/50; temperature: 250ºC) into a capillary column (Suprawax 280, 30 m, 0.53 mm internal diameter), programmed from 45ºC to 230ºC at 3ºC/min, final isotherm 30 min, with hydrogen as carrier gas (1 mL/min). The detection was performed with a flame ionization detector (FID) at 260ºC. Quantification was carried out by reference to a standard range prepared under the same conditions.

Analysis of the content ion silver in wines

Content of silver was determined at the end of the experiments in all wines using a Zeeman graphite furnace atomic absorption spectrometer Varian model AA240ZGTA 120 (Varian Inc. Walnut Creek, CA, USA), after ashing the sample and dissolving in nitric acid following the official analytical method OIV-MA-AS322-09 (OIV, 2016).

Statistical analysis

Data were subjected to the Student's t test and Student-Newman Keuls test to identify any statistically significant differences between treatments, using SPSS software (version 12.0).

Results and discussion

Inactivation of *Brettanomyces*

Figure 2 shows the evolution of the viable population of *Brettanomyces* in CFU/mL at 0, 3, 10, 17 and 24 days of the Trial 1. When the wine was inoculated with a population of 1.0E+2 CFU/mL (**Figure 2a**), a reduction almost 1 log of the initial population of *Brettanomyces* was observed in the wines treated with KAgC at day 3 of contact with the product. In the control wine, without KAgC, an increase of the population of *Brettanomyces* was detected from day 3, further increasing to 4 log on day 10. In wines with KAgC a small increase was also observed on days 10 and 17, probably due to the growth inertia of the vegetative cells caused by the growth of a culture in a synthetic medium (YPD). But this growth ceased and the population clearly decreased in the sample taken on day 24 of treatment, when the count in the wine with KAgC indicated a reduction of more than 2.5 log compared to the one conducted on day 17, more than 1.5 log compared to the baseline population and approximately 5 log compared to the control wine without KAgC.

Figure 2b shows the behavior of *Brettanomyces* when the initial population was about 1.0E+4 CFU/mL. The KAgC showed strong action at day 3 reducing almost 3 log with respect to the initial population. This inactivation was virtually total at day 10 from the start of treatment. After this sampling, the population of *Brettanomyces* remained well below the initial concentration of cells added to the wine: from 14000 CFU/mL at day 0 to only 49 CFU/mL at day 24 (reduction of 2.45 log).

In wines with a population of 1.0E+06 CFU/mL (**Figure 2c**) the level of inactivation of the *Brettanomyces* population of the previous experiments was not achieved, although a smaller population (between 1 and 1.5 log) was found on day 24 of treatment, while the control maintains the same initial population at 24 days. It should be mentioned that it is very difficult to find such high populations of *Brettanomyces* in real conditions, in naturally contaminated wines. In these cases, the results indicate that perhaps it would be necessary to treat the wines with doses of over 1g/L of KAgC.

Acetic acid is a parameter that could be indicative of contamination of wine with *Brettanomyces* (Garijo et al. 2017). **Table 2** shows the values of this parameter in wines from Trial 1 with different initial concentration of *Brettanomyces* cells, with or without KAgC

(control). Control wines were those in which acetic acid had a considerable increase over the 24 days of the trial, reaching values between 1.20 and 1.56 g/L of acetic acid.

In the case of wines inoculated with *Brettanomyces* populations of 1.0E + 02 CFU/mL and 1.0E + 04 CFU/mL where KAgC was added, no significant increases in the acetic acid were detected during the 24 days of the study. In the KAgC wines initially inoculated with populations of 1.0E+06 CFU/mL where high population of *Brettanomyces* cells were detected, the acetic acid also increased, a fact which demonstrates that the strains used in the trial produce acetic acid. In this case, higher concentrations of KAgC (above 1 g/L) or combination of KAgC with other antimicrobial substances or techniques may be required to stop *Brettanomyces* growth.

4-ethypl-phenol concentration was analyzed at the end of the trial (24 days) in wines contaminated with *Brettanomyces*. 4-ethyl-phenol in control wines (without KAgC) was higher than those where 1 g/L of KAgC was added, whatever the initial inoculum of *Brettanomyces* (**Table 2**). Values of this metabolite in control wines with 1.0E+04 and 1.0E+06 initial Brett cells exceeded the perception threshold 425 µg/L (Chatonnet et al. 1992). In wines treated with KAgC, although 4-ethyl-phenol was produced by *Brettanomyces* cells, never exceeded this threshold. The concentration of this metabolite in the samples with KAgC was 79% lower than the control wine starting from an initial inoculate of *Brettanomyces* of 10^2 CFU/mL, 95 % lower in the case of 10^4 CFU/mL and 55% less starting from a population of 10^6 CFU/mL *Brettanomyces*. These results show that KAgC was able to slow the growth and viability of *Brettanomyces* and, consequently, decrease the possibility of unpleasant odors being produced due to this contaminating yeast which would have a negative effect of the sensory profile of the wine.

In Trial 2, both KAgC and chitosan treatments allowed a significant reduction of *Brettanomyces* population (**Table 3**). Thus, when 1g/L of KAgC was added to the Tempranillo wine naturally contaminated with of 1.0 x 10^4 GU/mL (Genomic Units) of *B. bruxellensis,* populations declined to 1.2 x 10^2 GU/mL ten days after addition, however in the control wines at the same period, *B. bruxellensis* increased 0.57 log. When 7 g/HL of fungal chitosan was added to the same initial wine, populations of *B. bruxellensis* declined to 3.0 x 10^2 GU/mL ten days after addition. There were no significant differences between samples treated with KAgC or chitosan. Hence, according this data, both KAgC and chitosan would reduce, but would not eliminate, this spoilage yeast. Regarding to chitosan, similar results

were obtained by Petrova *et al* (2016) when they inoculated 8.8 x 10^5 CFU/mL of *B. bruxellensis* in a Merlot wine. In that trial, populations of *B. bruxellensis* declined to 10^2 CFU/mL eleven days after addition of 4 or 10 g/HL of fungal chitosan. Blateyron-Pic et al. (2012), in wines naturally contaminated with 10^5 CFU/mL of *Brettanomyces*, found a residual population 10 days after treatment with 4 g/HL of chitosan of near to 100 CFU/mL. Ferreira et al (2013) found that the anti-yeast activity of chitosan was strain dependent because when they inoculated 7 log CFU/mL of two *Brettanomyces* strains on a red wine from the Alentejo region of Portugal, one yeast strain was inactivated, while the other yeast strain was more resistant (3 log cycle reduction).

Therefore, according to the data obtained by the Q-PCR, both treatments would give the impression of being effective in reducing populations of *B. bruxellensis* in a naturally contaminated wine, but not to obtain the elimination them completely. It is therefore of interest to check the status of the *Brettanomyces* residual population after treatments with KAgC and chitosan.

Inactivation of acetic acid bacteria

Figure 3 shows the evolution of the population of acetic acid bacteria in CFU/mL at 0, 1, 2 and 3 days of the Trial 1. When the wine was inoculated with a population of 1.0E+2 CFU/mL (**Figure 3a**) the population of AAB fell by 2 log during the first day of treatment with KAgC and no culture-viable cells were detected after 3 days from commencement of the experiment. Although there was also a decrease in the control wine between T0 and T3 (1 log) due to the inhibitory effect that the wine itself has on AAB, this drop was not as great as in the samples with KAgC.

The evolution of the population with a baseline AAB concentration of 1.0E+04 CFU/mL (**Figure 3b**) was similar to the previous case. There was a reduction in the population of 1.89 log during the first 24 hours of contact with the KAgC complex. And 3.37 log after 48 hours. After the third day the inactivation of the AAB was complete in KAgC wines. In the control wine the population also fell slightly, 0.62 log in 3 days.

In wines with a population of 1.0E+06 CFU/mL (**Figure 3c**) the anti-bacterial effect of KAgC was detected on day one in the bottles which contained it, with a reduction of 1.9 log, the same as in the initial concentration of 1.0E+04 CFU/mL and very similar to that with 1.0E+02 CFU/mL. In the control wine, over the same period, the loss of viability was only

0.52 log. After 3 days contact with KAgC a decrease in AAB of 2.9 log was achieved while in the control batch, due to the effect of the master itself, the reduction was 0.97 log.

Similar results were obtained by Izquierdo-Cañas et al (2012) when a colloidal silver complex at doses of 1 g/Kg was applied in Merseguera and Monastrell musts, achieving a decrease between one and two orders of magnitude in AAB populations at the end of alcoholic fermentation. Garde-Cerdán et al (2013) compared the action of colloidal silver particles (KAgC) and SO_2 on viable AAB counts in the Tempranillo winemaking process in must and 24 h after treatment with SO_2 or KAgC and concluded that the addition of SO_2 did not affect de AAB population whereas the presence of KAgC reduced it by 2 log CFU/mL. Finally, García-Ruiz et al (2015) using silver-based, biocompatible nanoparticles to evaluate their antimicrobial activity against enological AAB, among other microorganisms, also demonstrated the efficiency of ion Ag in controlling microbial processes in winemaking.

In this study, the data showed that KAgC had a rapid antimicrobial effect on a group of wine spoilage microorganisms such as the AAB, achieving irrespective of the initial population a fall of almost 2 log during the first day of contact with KAgC and between 2.3 and 3.97 log reduction by the third day.

Moreover, the action of KAgC on these spoilage bacteria reveals an additional advantage compared to other alternatives to SO_2 in microbiological control, such as lysozyme, which only acts against gram-positive bacteria and not against gram-negative ones such as AAB.

As in the case of *Brettanomyces,* it would be necessary to test the effect of a treatment at a concentration of above 1 g/L of KAgC when the initial AAB concentrations were around 1.0E+06 CFU/mL.

In trial 2, both KAgC and chitosan treatments allowed a significant reduction of acetic acid bacteria population (**Table 3**). Thus, when 1 g/L of KAgC was added to a Tempranillo red wine naturally contaminated with populations of 1.1 x 10^5 GU/mL of acetic acid bacteria, populations declined to 1,72 x 10^3 GU/mL. Thus, as occurred with *Brettanomyces*, KAgC reduced acetic acid bacteria by 2 log GU/mL although it did not completely eliminate these spoilage bacteria. When 7 g/HL of fungal chitosan was added to the same initial wine, populations of acetic acid bacteria declined to 3.47 x 10^3 GU/mL ten days after addition. In this sense, when Valera et al. (2017) compared chitosan and SO_2 effects in artificially contaminated wines with two strains of the species of acetic acid bacteria *Acetobacter*, they detected that their viability decreased with the application of chitosan. In our study there

were no significant differences between samples treated with KAgC or chitosan. Hence, according this data, both KAgC and chitosan would reduce, but would not eliminate, these spoilage bacteria.

Concentration of ion Ag in final wines

Regarding silver content in the final wines, it was far below the legal limit of 100 µg/L (0.1 mg/L) established by the OIV-OENO 145-2009, (OIV, 2015). This corroborates the results by Izquierdo-Cañas et al (2012) who studied the application of colloidal silver complex in winemaking.

Conclusion

According to these results, the viability of the yeast *Brettanomyces bruxellensis* and AAB, the main microorganisms which can affect wines organoleptic features, reduced by the presence of KAgC. In the case of *Brettanomyces* and AAB with populations of 1.0E+06 CFU/mL, the effect is less marked and it would be necessary to test whether a greater concentration of KAgC would have the desired effect.

In the case of acetic acid producing strains of *Brettanomyces,* it has been shown that the presence of KAgC decreases the risk of their production although in the wine there may be small residual populations of this yeast. In the same way, the risk of producing 4-ethyl-phenol is decreased in the presence of KAgC correlated with the inactivation of the strains of *Brettanomyces* which produce this metabolite.

The effectiveness of KAgC in the reduction of populations of *B. bruxellensis* and AAB has been demonstrated by two methods of microbiological analysis: plate counts and Q-PCR. Its action on *Brettanomyces* cells in naturally contaminated wines was very similar to that achieved with Chitosan.

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Figure 1: Amplification of the internal transcribed spacers (ITS1 and ITS2) of the rRNA 5.8S of four *Brettanomyces bruxellensis* strains used in the study. Lanes 1 to 4: strain, 1, strain 2, strain 3, strain 4. MWM: molecular weight marker 100 pb Ladder.

Figure 2: Populations of *Brettanomyces* in wine artificially polluted along 24 days after KAgC treatment.

Figure 3: Populations of acetic acid bacteria in wine artificially polluted along 3 days after KAgC treatment.

Table 1: Physicochemical parameters of initial wines.

Table 2: Acetic acid and 4-ethylphenol concentration in wines artificially polluted with increasing amounts of *Brettanomyces*.

Table 3: Populations of *B. bruxellensis and* acetic acid bacteria (GU/mL) in wine naturally contaminated (or polluted) before and after ten days of treatments with KAgC and chitosan.

Table 1:

	Wine 1	Wine 2
Alcohol content (% v/v)	11.48	14.26
Volatile acidity (g/L of acetic acid)	0.50	0.76
Total acidity (g/L of tartaric acid)	6.40	5.35
pH	3.48	3.66
Glucose +Fructose (g/L)	0.20	0.25
Total SO_2 (mg/L)	4	85
Free SO_2 (mg/L)	n.d.	7
4-ethylphenol (μg/L)	n.d.	1087
4-ethylguaiacol (μg/L)	n.d.	146

n.d.: Not Detected.

Table 2

Table 2:

Initial CFU/mL	1.0E+02		1.0E+04		1.0E+06	
Time (Days)	Control	KAgC	Control	KAgC	Control	KAgC
Acetic acid (g/L)						
0	0.51 ± 0.01[a]	0.51 ± 0.01[a]	0.50 ± 0.01[a]	0.50 ± 0.01[a]	0.49 ± 0.01[a]	0.50 ± 0.01[a]
24	1.20 ± 0.30[b]	0.53 ± 0.01[a]	1.56 ± 0.15[b]	0.61 ± 0.20[a]	1.50 ± 0.09[b]	1.39 ± 0.03[b]
4-ethylphenol (μg/L)						
0	32.5 ± 5.2[a]	29.1 ± 6.4[a]	34.2 ± 4.3[a]	30.7 ± 6.1[a]	33.8 ± 4.6[a]	33.8 ± 4.6[a]
24	143.4 ± 10.6[b]	30.1 ± 5.8[a]	516 ± 19.1[b]	26.3 ± 7.8[a]	785 ± 50.8[b]	352 ± 31.8[b]

Different superscripts [(a, b, c)] in the same column indicate significant differences for α = 0.05 according to the Student- Newman-Keuls test.

Values are the mean of triplicates.

Table 3:

	Brettanomyces		Acetic acid bacteria	
	Initial wine	10 days after treatment	Initial wine	10 days after treatment
Control	1.00E+04	3.73E+04[b]	1.10E+05	8.27E+05[b]
KAgC		1.17E+02[a]		1.72E+03[a]
Chitosan		3.00E+02[a]		3.47E+03[a]

Different superscripts [(a, b,)] indicate significant differences in the same column for α = 0.05 according to the Student- Newman-Keuls test. Values are the mean of triplicates.

Figure 1

Figure 1:

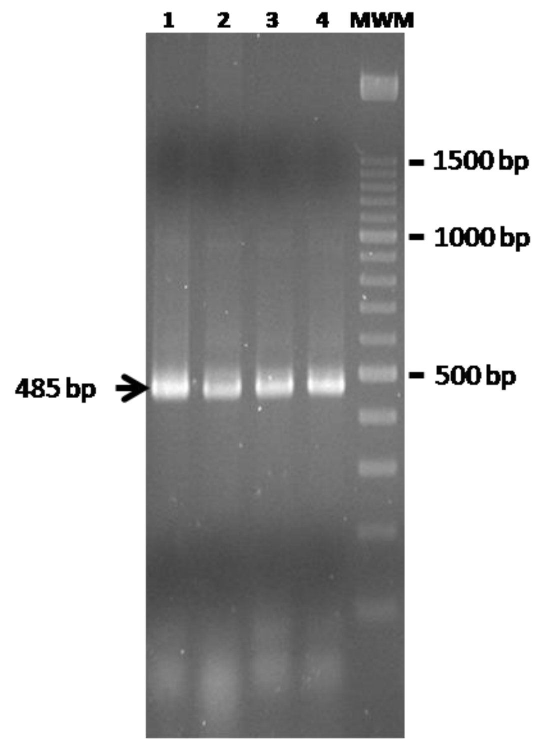


Figure 2

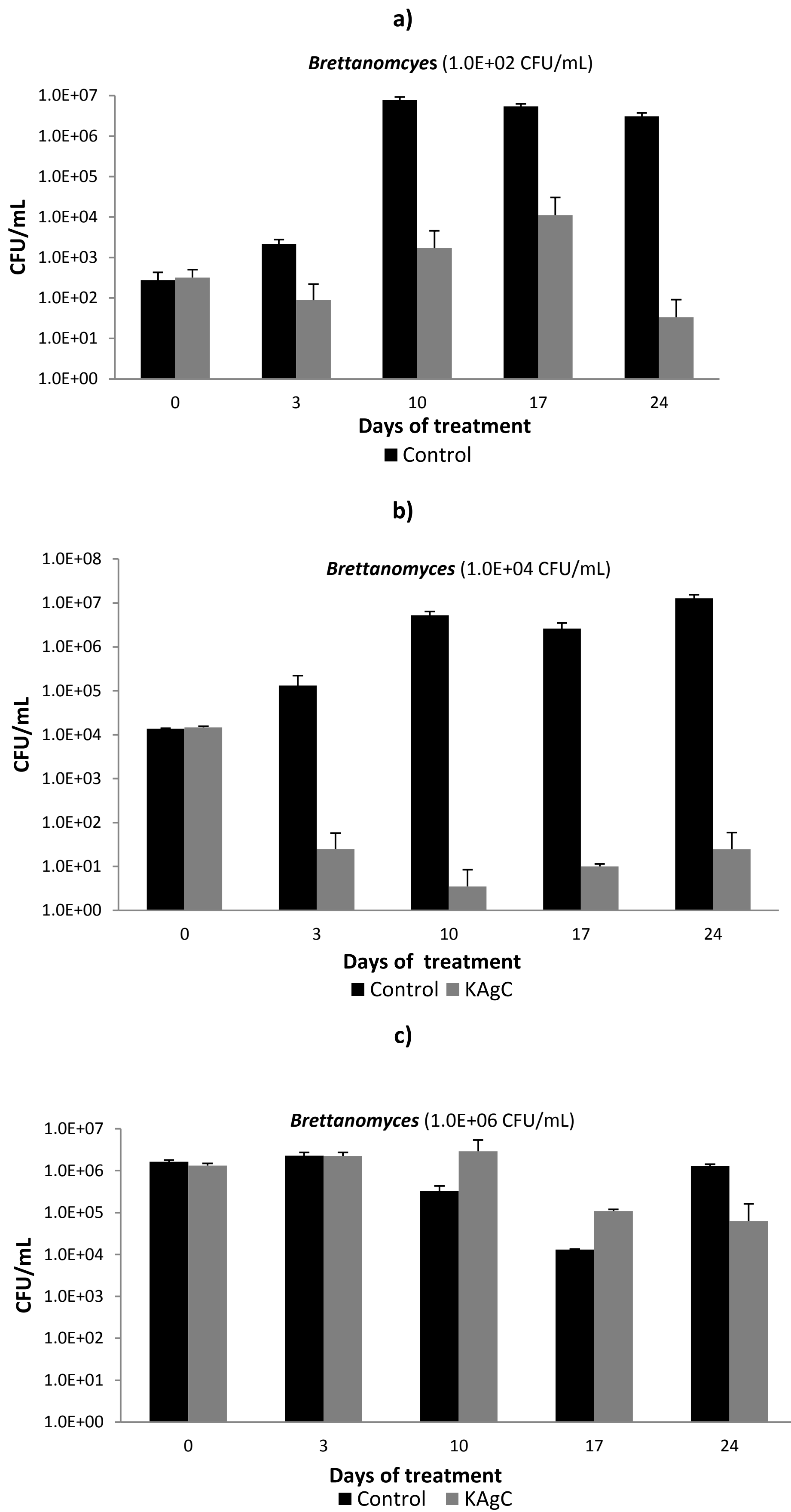
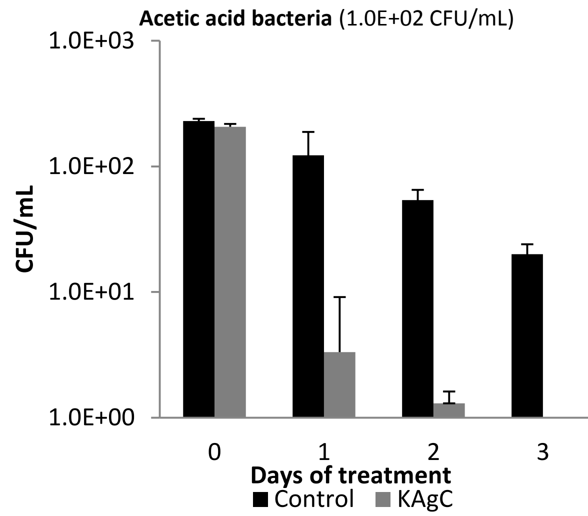


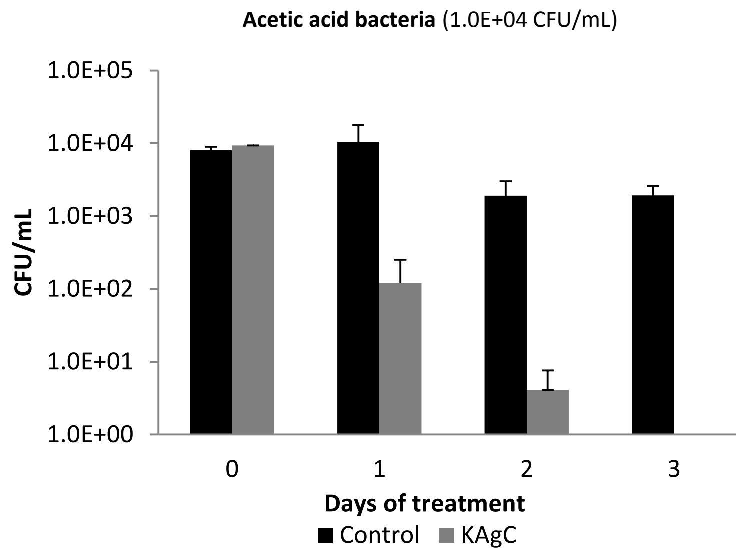
Figure 3

Figure 3:

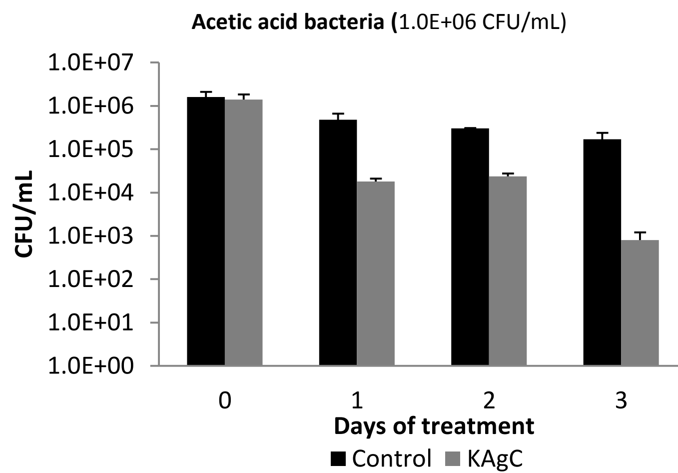
a)



b)



c)



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