Improved antimicrobial activity of immobilised essential oil components against representative spoilage wine microorganisms

Estéfani García-Ríos[a], María Ruiz-Rico[b]*, José M. Guillamón[a], Édgar Pérez-Esteve[b], José M. Barat[b]

[a] Departamento de Biotecnología de los alimentos, Instituto de Agroquímica y Tecnología de los Alimentos (CSIC), Avda. Agustín Escardino, 7, E-46980-Paterna, Valencia, Spain

[b] Departamento de Tecnología de Alimentos, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

Corresponding author Present address: Grupo de Investigación e Innovación Alimentaria. Departamento de Tecnología de Alimentos, Universitat Politècnica de València. Camino de Vera s/n, 46022, Valencia, Spain. Tel: +34 963877000 ext. 83612. *E-mail address*: maruiri@etsia.upv.es

Abstract

Wine, as a fermented drink, is considered a microbiologically safe beverage, but the growth of spoilage microorganisms can cause economic damage. As a new preservative process, the application of immobilised essential oil components (EOCs) is proposed in this study. EOCs were attached to the surface of three different commercial supports (silica particles, cellulose particles and cellulosic membrane) to avoid the disadvantages of using these compounds in their free form, such as volatility, low water solubility and intense aroma. The results showed that the treatment of spoilage microorganisms with antimicrobial particles (silica and cellulose) significantly reduced the viability and growth capacity of the target microorganisms. The covalent attachment of EOCs to particles led to a significant reduction in both the MIC values and viability compared with most free compounds. The enhanced antimicrobial activity of EOCs after their anchorage to a support was confirmed, resulting in MIC values of 10-90 fold lower than those of the free bioactive compounds. In addition, the filtration of microorganism suspensions through EOC-functionalised membranes showed remarkably antimicrobial activity.

Keywords: carvacrol; cellulose; eugenol; immobilisation; naturally-occurring antimicrobial; silica; thymol; vanillin.

1. Introduction

Wine is produced through a complex biochemical process that involves interactions among fungi, yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) that start in the vineyard and continue throughout the fermentation process until packaging (Fleet, 2003). Some of these microorganisms are responsible for the transformation of grape must into wine and have to be eradicated owing to their spoilage ability; i.e., friends or foes, depending on the winemaking stage. Among the most frequent wine spoilage yeasts, *Brettanomyces bruxellensis*, or its teleomorph known as *Dekkera bruxellensis*, the osmotolerant *Zygosaccharomyces bailii* or the main wine species *Saccharomyces cerevisiae* must be considered (Du Toit & Pretorius, 2000; Fleet, 2003).

The main defects produced by fermentative species, such as *S. cerevisiae* and *Z. bailii*, are refermentations in sweet wines or with residual sugar, which leads to large amounts of CO_2 and high turbidity in bottled wine. Moreover, *Z. bailii* is very resistant to most of the antimicrobials used in wine, such as sulphur dioxide (SO_2), sorbate, etc. (Kuanyshev et al., 2017). Nowadays however, the main nightmare for oenologists is represented by *B. bruxellensis*, which is able to grow during wine ageing and spoils wines in cellars. This species produces volatile phenols from phenolic acids, such as p-coumaric and ferulic acids, that negatively impact wine aroma (Smith & Divol, 2016). Despite the positive effect of some lactic acid bacteria species during malolactic fermentation, these microorganisms produce a vast number of wine defaults (Lonvaud-Funel, 1999). Finally, the last main spoilage bacteria group in wine is acetic acid bacteria. These aerobic bacteria can, in any case, be considered desirable microbiota because their oxidative metabolism mainly transforms sugars and ethanol into acetic acid and ethyl acetate, which increases the so-known volatile acidity, and confers wine an undesirable aroma (Guillamón & Mas, 2017).

Thus wine spoilage microbes are those microorganisms found in the wrong place at the wrong time, including microbes that are normally desirable and contribute to the quality of the end product. The point at which any microorganism is undesirable is perhaps bottled wine. The control of wine stability throughout production and after bottling is a major concern for the wine industry. This microbiological control is carried out in wineries by chemical and physical treatments. Among chemical preservatives, SO_2 is the most widespread and universal antimicrobial compound used in oenology to prevent wine spoilage. It is also an antioxidant and limits the growth of indigenous microbiota (Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008). However, winemakers attempt to reduce SO_2 doses due to adverse reactions in humans, including allergies to sulphites and sulphates (Bartowsky, Xia, Gibson, Fleet, & Henschke, 2003). Regarding physical stabilisation techniques, although wine filtration by sterilising membranes (0.45 µm) is the most widespread practice in wineries, intense filtration impacts the organoleptic and structural properties of treated wines (Arriagada-Carrazana, Sáez-Navarrete, & Bordeu, 2005).

Currently, wine consumers prefer less processed wines that maintain their high quality. To meet this demand, new preservative agents or stabilisation techniques need to be developed. Natural products from various organisms capable of preventing microbial spoilage can be an alternative to using chemical products (Petrova, Cartwright, & Edwards, 2016; Ruiz-Rico et al., 2017). Naturally-occurring antimicrobial compounds, such as essential oil components (EOCs), have been investigated as appropriate biocompatible antimicrobial agents for treating alterative food microorganisms (Dunn, Davidson, & Critzer, 2016; Prakash, Kedia, Mishra, & Dubey, 2015; Rupasinghe, Boulter-Bitzer, Ahn, & Odumeru, 2006). However, some features of these antimicrobial compounds, such as their strong sensory properties (odour and flavour), poor water

solubility and instability (Burt, 2004; Shah, Davidson, & Zhong, 2012; Turek & Stintzing, 2013), restrict their application to food products.

Alternative technologies for the direct application of EOCs have been recently proposed to prevent the above-mentioned limitations. Among them, inert materials like clay, silica or cellulose can be used as materials for the encapsulation (Bernardos et al., 2015; Ruiz-Rico et al., 2015), immobilisation of bioactive compounds onto their surface (Cappannella et al., 2016; Ribes et al., 2017; Ruiz-Rico et al., 2017) or nanoemulsion (Sow, Tirtawinata, Yang, Shao, & Wang, 2017).

Despite both strategies fulfilling the goal of creating new devices that improve the antimicrobial characteristics of essential oils, the chemical immobilisation of EOCs onto the surface of a particle allows the noticeable and strong aromas of these bioactive compounds to be masked.

Accordingly, this study aims to develop a collection of antimicrobial agents based on the immobilisation of several volatile EOCs (carvacrol, eugenol, thymol and vanillin) onto the surface of two types of materials with different chemical reactivities (cellulose and SiO_2), and to evaluate their antimicrobial activity against some wine spoilage microorganisms, e.g., *S. cerevisiae*, *Z. bailii*, *D. bruxellensis*, *L. plantarum* and *A. aceti*, compared with that of free bioactive compounds.

2. Material and Methods

2.1 Chemicals

(3-Aminopropyl) triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butanone, dimethyl sulfoxide (DMSO), carvacrol, eugenol, thymol, sodium borohydride and microcrystalline cellulose particles were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile, HCl, H_2SO_4, KOH and $MgSO_4$ were provided by

Scharlab (Barcelona, Spain). Vanillin was obtained from Ventós (Barcelona, Spain). Amorphous silica microparticles were supplied by Silysiamont (Milano, Italy).

2.2 Synthesis of EOC-functionalised particles

Antimicrobial supports were synthesised following the methodology previously described by Ruiz-Rico et al., (2017) with some modifications. Firstly, the aldehyde derivatives of carvacrol, eugenol and thymol were produced by adding a second moiety capable of reacting with APTES to synthesise alkoxysilane derivatives. This step is needed to maintain the hydroxyl group free of EOCs, which is responsible for the antimicrobial activity of bioactive compounds (Gill & Holley, 2006; Hyldgaard, Mygind, & Meyer, 2012).

Carvacrol and thymol aldehydes were synthesised by direct formylation using paraformaldehyde. To this end, 40 mmol of carvacrol or thymol and 40 mmol of anhydrous $MgSO_4$ were placed in a round-bottomed flask in an argon atmosphere. Next 150 ml of acetonitrile and 150 mmol of trimethylamine were added to the flask and the mixture was stirred for 15 min at room temperature. Then 270 mmol of paraformaldehyde were added and the reaction mixture was refluxed for 3.5 h at 83ºC. After cooling the reaction, the solution was acidified with 5% HCl solution and stirred for 30 min in an inert atmosphere. Finally, the organic portion was extracted with diethyl ether and volatiles were removed under reduced pressure.

Eugenol aldehyde was synthesised following a Reimer–Tiemann reaction. In a typical synthesis, 22 mmol of eugenol were dissolved in 150 ml of water at 80°C in a round-bottomed flask. After cooling the mixture to 60°C, 400 mmol of KOH and 88 mmol of chloroform were added (chloroform was added at a rate of 1 ml/h over a 7-hour period for safety reasons). The mixture was kept at 60ºC for 8 h and the solution was acidified

with 10% H_2SO_4 solution. The organic portion was extracted with n-butanone and volatiles were removed under reduced pressure.

In a second step, the alkoxysilane derivatives of EOCs were synthesised by the reaction of the aldehydes of carvacrol, eugenol and thymol and pure vanillin with (3-aminopropyl) triethoxysilane. In a typical synthesis, the EOC aldehyde derivatives and unmodified vanillin were suspended in dichloromethane, and APTES was added to the mixture at a molar ratio of 1 EOC:1 APTES. The mixture was stirred in reflux for 1 h and evaporated under reduced pressure to obtain the corresponding alkoxysilane derivatives.

The third step consisted of anchoring the alkoxysilane derivatives to the surface of two different supports: amorphous silica particles and cellulose particles. For this purpose, 1 g of bare silica or cellulose particles was placed in a round-bottomed flask in an inert atmosphere. Then 40 ml of acetonitrile or DMSO were added to the flask for the silica or cellulose microparticles, respectively. After particles had been suspended, an excess of alkoxysilane derivatives was added and the final mixture was stirred for 5.5 h at room temperature. Once the EOCs had attached to the surface of particles, reduction of the imine bond formed between the aldehyde group of EOCs and the amine group of APTES was performed to stabilise the chemical bond of the immobilised EOCs. Solids were centrifuged and suspended in 40 ml of methanol, and an excess of sodium borohydride was added. The mixture was stirred for 12 h. Finally, solids were centrifuged, washed with methanol and distilled water (pH 4), and dried at room temperature in vacuum for 12 h.

2.3 Synthesis of the EOC-functionalised paper membranes

The EOC-functionalised membranes were synthesised following the above-described procedure with some changes. For this purpose, a solution of alkoxysilane derivatives

(50% v/v) in dimethyl sulfoxide was prepared. Then 1.5 ml of the mixture was added drop by drop to the paper membranes (⌀ 4.5 cm). Once the membranes were well impregnated, they were dried at room temperature for 2 h. The functionalised membranes were immersed in a solution of methanol with sodium borohydride for 1 h to stabilise the chemical bond of the immobilised EOCs. Finally, membranes were washed with an excess of distilled water (pH 4) and were dried at room temperature for 24 h.

2.4 Materials characterisation

The bare and functionalised supports were characterised by standard techniques: field emission scanning electron microscopy (FESEM), particle size distribution, zeta potential and determination of the degree of functionalisation by an elemental analysis. FESEM images were acquired by a SUPRA® 25 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. Particle size distribution was established in distilled water using a Malvern Mastersizer 2000 (Malvern Instruments, UK). Suspensions of particles were previously sonicated to avoid the agglomeration of solids. All the measurements were taken in triplicate. The zeta potential (ζ-potential) was determined by a Zetasizer Nano ZS (Malvern Instruments, UK). The solids suspended in distilled water (1 mg/ml concentration) were previously sonicated to preclude aggregation. The zeta potential was calculated from the particle mobility values with the Smoluchowski model. The elemental analysis (C, H, O and N) was done by a combustion analysis in a CHNOS model Vario EL III (Elemental Analyses System GMHB, Langenselbold, Germany). In addition to standard techniques, the bulk density of the silica and cellulose particles was determined by pouring a fixed amount of particles into a graduated cylinder and recording the volume occupied after compacting the solids. By

taking into account the obtained data from the characterisation, the amount of EOCs (mg/cm^2) grafted to the different support's surface was estimated (Ruiz-Rico et al., 2017).

2.5 Microbiological analysis

In this study, five strains of bacteria and yeast related with the spoilage of a number of beverages and food were used: *L. plantarum* (*Lp*), *A. aceti* (*Aa*), *D. bruxellensis* (*Db*), *Z. bailii* (*Zb*) and *S. cerevisiae* (*Sc*). The details of all the strains are found in Table S1. Inoculum were prepared by introducing one single colony from each strain into 10 ml of MRS (Scharlau) for bacteria or SC (2% glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate and 0.074% complete supplement mixture (Difco)) for the yeasts. The mixture was incubated at 28 ºC for 24 h to obtain an inoculum with a density of approximately 10^8 cells/ml of broth.

2.5.1 Antimicrobial susceptibility assays

Different cultures of the five tested microorganisms were appropriately diluted to reach a density of approximately 10^6 cells/ml in 15 ml of PBS with different free or immobilised antimicrobial concentrations: 0, 0.025, 0.05, 0.125, 0.25 and 0.5 mg/ml. The flasks with the different microorganisms and antimicrobial concentration were incubated with orbital stirring (150 rpm) at 28 ºC for 24 h and were closed with cotton. All the treatments were performed in triplicate. Positive and negative controls were included in all the assays.

After incubation, viable cell numbers were determined as colony-forming units (CFU) by plating in different culture media (MRS for bacteria, SC for yeasts) and were incubated at 28 ºC for 48 h. In order to assess the impact of EOCs on the growth of survival cells, the same samples were washed to remove the antimicrobial compounds and an initial density of approximately 10^6 cells/ml was inoculated in fresh medium (SC or MRS). The

effects on the growth rate were monitored by determining optical density (OD) at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 28 ºC. Measurements were taken every 30 min for 2 days after 20-s pre-shaking for all the experiments. Microplate wells were filled with the required volume of inoculum and 0.25 ml of SC or MRS medium to always ensure an initial OD of approximately 0.1 (inoculum level of approximately 10^6 cells/mL). For each experimental series, non-inoculated wells were also included in the microplate to determine and, therefore, to subtract the noise signal. All the experiments were carried out in triplicate. Growth parameters were calculated from each treatment by directly fitting OD measurements *vs*. time to the reparametrised Gompertz equation proposed by Zwietering et al., (1990):

$$y = D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D = \ln(OD_t/OD_0)$ is the asymptotic maximum, μmax is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h) (Aguilera, Randez-Gil, & Prieto, 2007). The overall yeast and bacteria growths were estimated as the area under the OD *vs.* the time curve (48 h). This parameter was calculated by integration using the OriginPro 8.5 software (OriginLab Corp., Northampton, MA, USA).

The effect of increasing bioactive compound concentrations on microorganism μ_{max} was well fitted by means of a linear regression fit:

$$y = -A*(x)+B$$

where y is the μ_{max} for each concentration (x), B is the μ_{max} in the absence of antimicrobial, and A is a slope parameter (this being negative due to the inhibitory effects of the bioactive compounds). The minimum inhibitory concentration (MIC) is the minimum value of x, where μ_{max} is 0, and represents the minimum antimicrobial concentration

where microorganisms are unable to grow (García-Ríos, Gutiérrez, Salvadó, Arroyo-López, & Guillamon, 2014; Salvadó, Arroyo-López, Barrio, Querol, & Guillamón, 2011).

2.5.2 Functionalised membranes analysis

Different cultures of the five tested microorganisms were appropriately diluted to reach a density of approximately 100-300 cells in 50 ml of PBS. These cellular suspensions were filtered through a system with two different filters: above, a cellulosic membrane coated with the different EOCs or without any bioactive compound for the control condition; below, a cellulose membrane (47 mm in diameter with a 0.45-µm pore size; Millipore, Merck), which retained the microorganisms contained in the sample. The second membrane was incubated in a suitable culture medium (SC and MRS) at 28 ºC for 2-3 days. The developed colonies were counted and the results were expressed as percentage of survival (compared with the control condition).

2.6 Determination of bacterial viability and agglomeration by fluorescence assay

A two-colour fluorescent assay, LIVE/DEAD® BacLight™ (Life Technologies, Gaithersburg, MD, USA), was used to visualise the viable and dead microbial cells. The kit provides a two-colour assay of bacterial viability. SYTO 9 (green-fluorescent nucleic acid stain) labels all microbial cells with either intact or damaged membranes. In contrast, propidium iodide (red-fluorescent nucleic acid stain) penetrates only the microorganisms with damaged membranes, which brings about a reduction in SYTO 9 stain fluorescence when both dyes are present.

The two provided dye components were mixed at a ratio of 1:1. Next 0.8 µl of SYTO 9/propidium iodide were added to 500 µl of the treated suspension, and were mixed and incubated for 10 min to facilitate the penetration of dyes. Then 5 µl of stained cells were

applied to poly-L-lysine-covered slides for immunofluorescence (Sigma-Aldrich, Madrid, Spain), and a coverslip was placed over the suspension and sealed. The preparation was incubated for 5-10 min at room temperature in the dark to allow microorganisms to adhere to slides. Slides were then observed under a Motic BA310E trinocular microscope equipped with an Epi-Led module, MB barrier filter and a Moticam 3+ camera.

2.7 Statistical analysis

All the experiments were carried out at least in triplicate. Physiological data were analysed by the Sigma Plot 13.0 software, and the results were expressed as mean and standard deviation. Significance was determined by an analysis of variance (ANOVA) using version 7.0 of the Statistica software package. The statistical level of significance was set at a *P*-value of ≤ 0.05 with a Tukey test. Phenotypic data were fitted to the reparametrized Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function of the R statistical software, v.3.0. Heatmaps were created using the heatmap3 package in the R statistical software, v.3.0.

3. Results

3.1 Material preparation and characterisation

Essential oil components were immobilised onto the surfaces of three different commercial supports to avoid the disadvantages of using bioactive compounds in their free form, such as volatility, low water solubility and intense aroma. Two of these surfaces, provided from the inert particles and considered GRAS (generally recognised as safe) materials and authorised as food additives, were amorphous silica microparticles

(E-551) and cellulose microparticles (E-460), inorganic and organic supports, respectively. The third surface was a cellulosic paper conventionally used to create filters. After the immobilisation of the different EOCs onto the surface of the three supports, the materials were characterised according to their nature (powder or tissue).

Figure S1 shows the morphological characterisation of the bare and EOC-functionalised materials performed by field emission scanning electron microscopy (FESEM) using vanillin-functionalised supports as reference supports. As this figure depicts, bare amorphous silica particles came over as irregular spheres with a rough morphology, while bare cellulose particles appeared like asymmetrical prisms with an irregular surface. No changes on the surface of the supports were detected when comparing the bare and functionalised particles, which confirmed that the immobilisation process did not affect the integrity of the supports.

The fibrillary structure of the EOC-functionalised paper membranes was observed by FESEM. Figure S2 shows the microstructure of the non-modified and vanillin-functionalised cellulose membranes. Both membranes present a similar configuration of cellulose fibres to form fibre gaps of up to 10 µm. This configuration allows microorganisms to pass through the pores between fibres at a reasonably fast gravity flow, which might favour direct contact between bacteria and the immobilised EOCs.

After morphological characterisation, particle size and the ζ-potential were determined in the particulated systems. Table S2 summarises the particle size distribution ($d_{0.5}$) and the zeta potential values of the bare and functionalised materials in distilled water. The bare and functionalised amorphous silica particles had a particle size on the microscale that was similar to the reported single particle size. Non-significant differences were found for the particles functionalised with the different bioactive compounds. The cellulose particles displayed a significantly larger particle size than the silica particles. Likewise,

no differences were observed between the $d_{0.5}$ values of the bare and functionalised cellulose particles.

The bare silica and cellulose materials had negative ζ-potential values due to the presence of SiO^- and COO^- groups, respectively. The ζ-potential of the EOC-functionalised particles changed in both cases to positive values, which confirmed the attachment of bioactive compounds onto the surface of both materials.

After the characterisation of the supports, the EOC density on the three supports' surfaces was estimated by an elemental analysis to study the influence of the amount of immobilised EOC on the antimicrobial properties of the developed antimicrobial agents. Table S3 shows the calculation of the density of the immobilised bioactive agents (mg/cm^2) on the surface of the silica and cellulose particles, and the cellulosic membranes, according to the characterisation results. As seen in Table S3, the amount of grafted EOC was significantly bigger for the cellulose particles than for the silica particles. Cellulosic membranes presented the highest density of the bioactive compounds, probably due to the material functionalisation methodology and the larger surface per gram of support.

3.2. Antimicrobial activity of EOCs free and immobilised on microparticles

To assess the antimicrobial activity of the developed particle-based systems, five strains of bacteria and yeast related to wine spoilage were used (*L. plantarum*, *A. aceti*, *B. bruxellensis*, *Z. bailii* and *S. cerevisiae*). For this purpose, cells were incubated in PBS for 24 h in the presence of different concentrations of the free and immobilised EOCs. The supports used for immobilisation, SiO_2 and cellulose, were also tested as negative controls. After incubation, viable cells were determined as CFU and the effect on the growth rate was determined by monitoring OD at 600 nm.

3.2.1 Effect on viability

Antimicrobial activity was evaluated by measuring the viability of the culture after incubation in presence of antimicrobials (free or immobilised). In order to gain a general overview of the multiple tested conditions, the average viability value in all the concentrations assayed for each compound and each microorganism was represented in a heatmap (Figure 1). Values close to 1 (yellow) mean higher viability values (less compound effectiveness), while values close to 0 (pink) mean lower culture viability (greater compound effectiveness), compared with the control condition (no antimicrobial treatment).

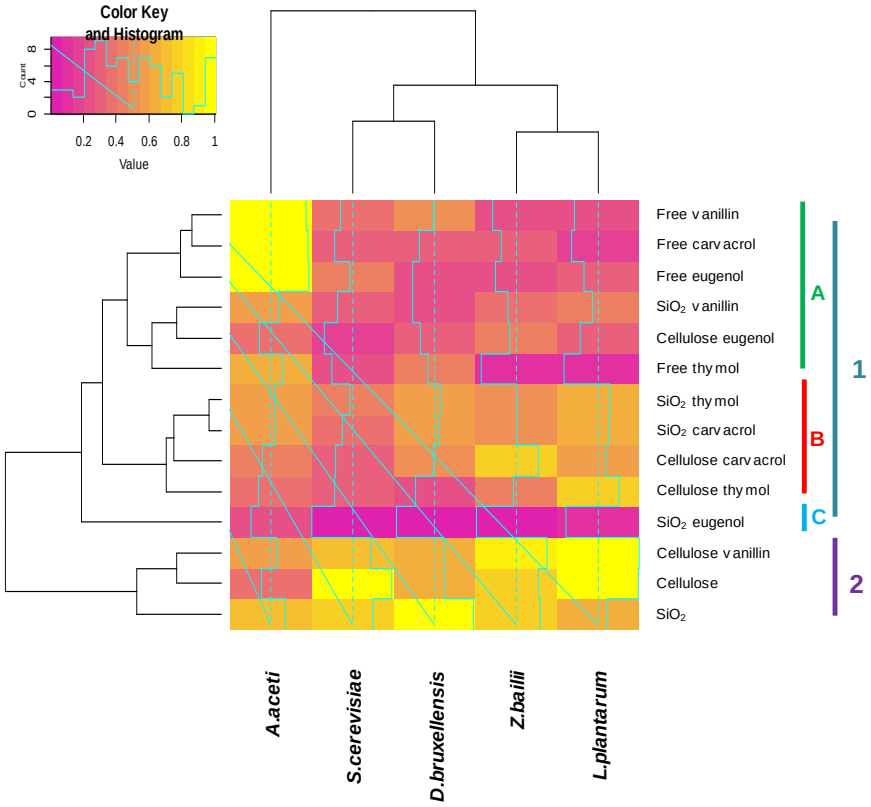

Figure 1. Mean viability values for each condition and each tested microorganism. Values below 1 mean that viability is affected compared with the control strain. Sensitivity is indicated in purple, and resistance in yellow. The three groups separated by the hierarchical clustering analysis (HCL) are marked by colours. The histogram shows the number of conditions located within the same growth range.

Hierarchical clustering divided the assayed conditions into two major groups (1 and 2). Group 1 was integrated by three subclusters (A, B and C) according to their antimicrobial potential. Cluster A grouped to the free EOCs, together with SiO_2 vanillin and cellulose eugenol, which showed good effectiveness by reducing the viability of cultures. Cluster B was formed mainly by EOCs with intermediate antimicrobial activity and strong strain-dependency. Cluster C was formed only by SiO_2 eugenol, which was the compound that provoked the higher mortality in all the microorganisms. Finally, the compounds with the highest viability values and, therefore, less effectiveness, made up Group 2. In this group, we found the bare particles and the vanillin immobilised in cellulose. Considering all these results, SiO_2 vanillin, cellulose eugenol, and mostly SiO_2 eugenol, proved the most effective immobilised EOCs in all the microorganisms of our study. Thymol showed the greatest effectiveness of the free EOCs.

Regarding strains, *Aa* proved to be the most resistant microorganism for all the tested conditions, especially for the free EOCs, and was clearly separated from the other strains in the cluster analysis. *Sc* and *Db* formed an independent cluster of *Lp* and *Zb*. These clusters indicated a similar susceptibility for the same compounds and concentrations. Yet at a glance, these four strains showed similar sensitivity to EOCs, with some compounds being very lethal for most strains.

In order to obtain more details of each assayed condition, Figures 2 and 3 represent the relative viability of the five microorganisms, compared with the control condition, for the different concentrations (0, 0.025, 0.05, 0.125, 0.25 and 0.5 mg/ml) of the EOCs immobilised in the silica and cellulosic particles, respectively. In the silica particles (Fig. 2), eugenol provoked the highest mortality in all five microorganisms, with a 100%decrease of viable population for the different concentrations. In fact practically 100% of the population of all the strains died at the 0.125 mg/ml concentration, except

for *Zb*, which needed 0.250 mg/ml for its viability to reduce by 100%. Conversely, none of the three remaining antimicrobials was able to cause 100% of mortality, except for the 5 mg/ml vanillin concentration, at which strongly reduced viability was also shown. In general, carvacrol was the least effective compound for all the assayed microorganisms, followed by thymol.

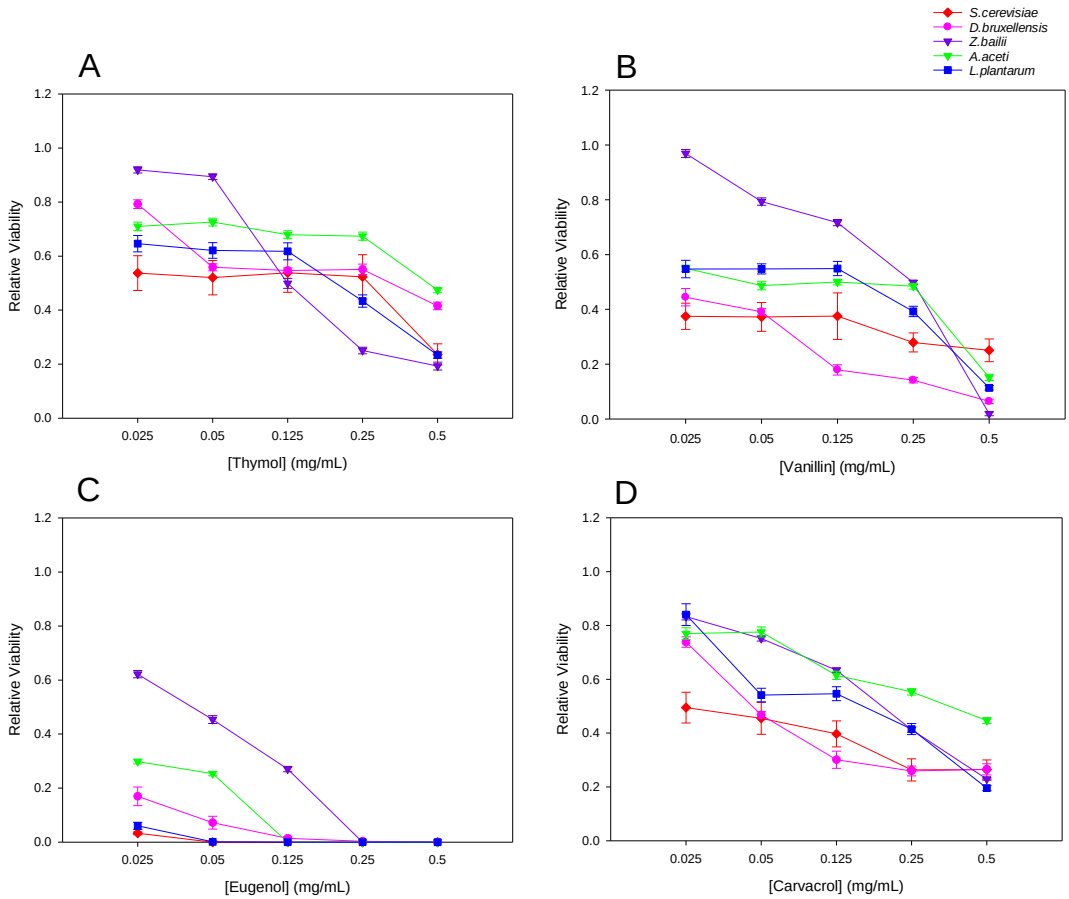

Figure 2. Relative viability of the five microorganisms used after incubation with thymol (A), vanillin (B), eugenol (C) and carvacrol (D) immobilised in the silica particles according to EOC concentration (means and standard deviations, n=3). The values are relative compared with the control condition without treatment.

For the cellulose particles (Figure 3), eugenol also proved to be the most effective EOC with percentage of less than 50% viability for all the microorganisms at 0.125 mg/ml. Besides eugenol, thymol was the next EOC with the lowest viability values, especially

for *Aa*, which showed greater resistance to most of the tested antimicrobial compounds. The 0% viability value was reached only in *Sc* with eugenol and *Zb* and *Aa* with thymol. These results agree with the estimated amount of EOC immobilised on the particles' surface since the systems with highest density of EOC were more effective (see Table S3). It was noteworthy that this landscape changed when viability was assessed after the incubation with the free EOCs (Fig. S3). Eugenol lost part of its effectiveness, which was especially remarkable for some strains, like *Aa*, which was completely unaffected by any of the tested concentrations, and thymol showed greater effectiveness than when immobilised, which led to a higher mortality at lower concentrations.

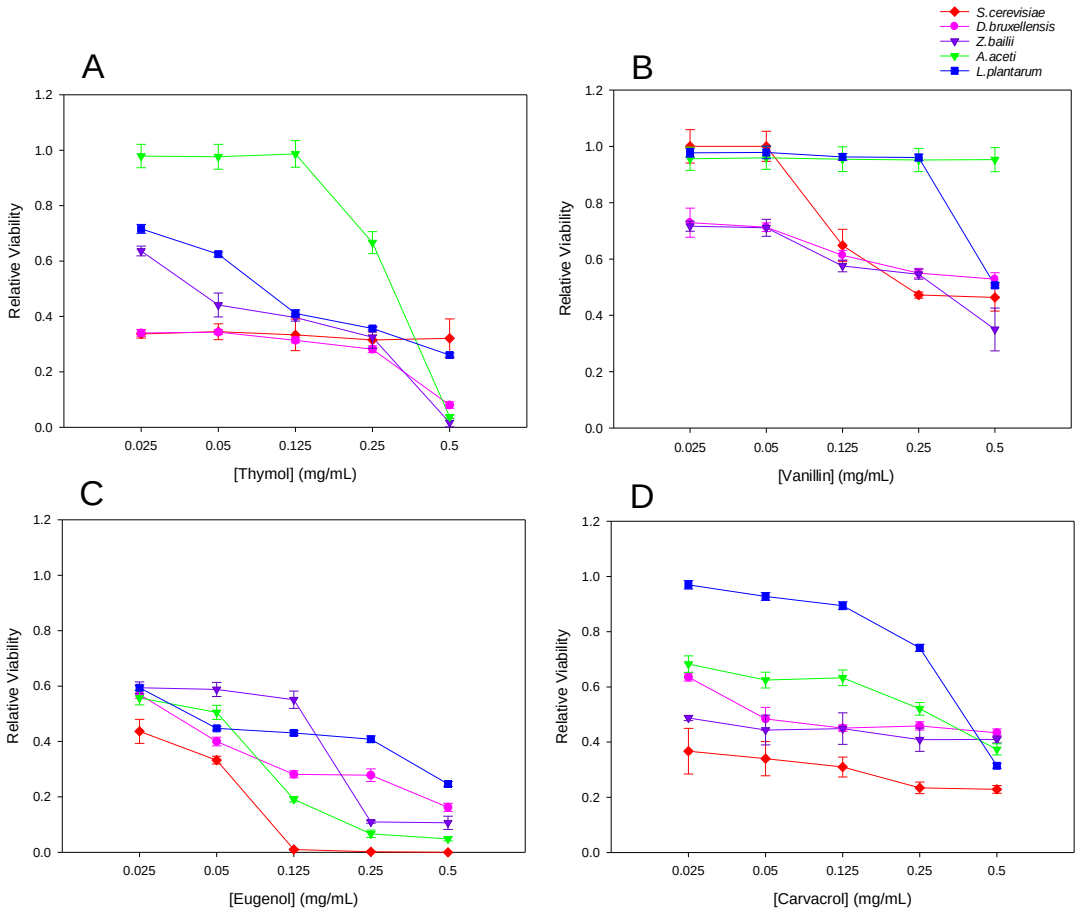

Figure 3. Relative viability of the five microorganisms used after incubation with thymol (A), vanillin (B), eugenol (C) and carvacrol (D) immobilised in the cellulose particles according to EOC concentration (means and standard deviations, n=3). The values are relative compared with the control condition without treatment.

We also tested the possible antimicrobial effect of the bare silica and cellulose particles to ensure that the antimicrobial activity only lay on EOCs (Figure S4). Although we observed a sharp drop in viability after the 24-hour incubation, mainly for the cellulose particles, we suspected that it could be due to microorganisms aggregating around particles. This phenomenon of aggregation and sedimentation of cells with particles was confirmed by analysing the viability of the cells incubated with the bare and functionalised particles by fluorescence microscopy. By way of example, Figure S5 shows pictures of the cells incubated in the presence of bare cellulose particles and the same particles functionalised with eugenol. As can be seen, 100% of the cells incubated in the presence of the bare particles were alive (green), whereas many cells died when they were incubated with the cellulose functionalised with eugenol (red).

3.2.2 Effect on growth capacity

In order to assess the effect of EOCs on the survival of cells after the 24-hour incubation, these cells were washed to remove antimicrobial particles, inoculated in fresh medium and their growth was monitored in a microtiter reader. The area under the different growth curves (AUC) was calculated to detect differences in growth behaviour. The values of these AUC are represented in Figure 4 as a heatmap, together with a hierarchical clustering (HCL) between samples. Values close to 1 (yellow) mean unaffected growth and, therefore, a low impact of EOC, while values close to 0 (pink) mean a high impact with impaired growth shown.

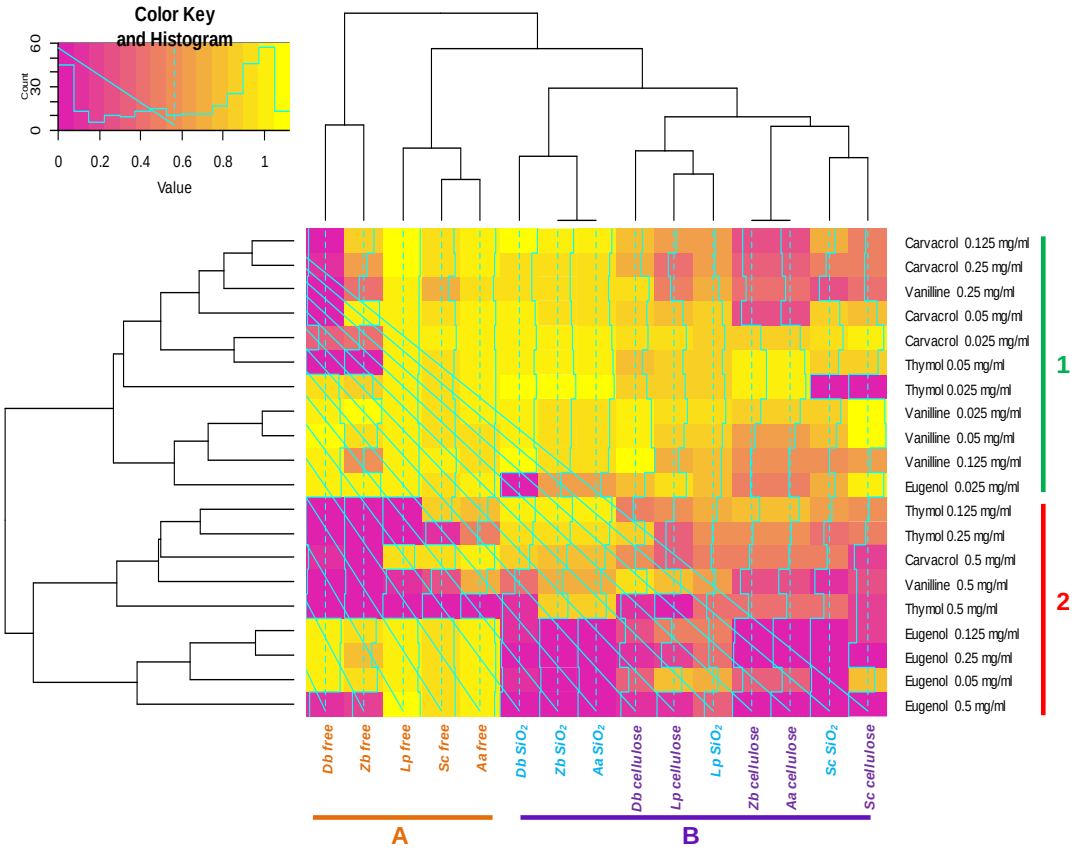

Figure 4. The area under the curve (AUC) of the five microorganisms for all the assayed conditions. The values are relative compared with the control condition without treatment. Values below 1 mean that the parameter is affected compared with the control strain. Sensitivity is indicated in purple, and resistance in yellow. The three groups separated by the Hierarchical clustering analysis (HCL) are marked by colours. The histogram shows the number of conditions located within the same growth range.

This heatmap compiles information about a huge amount of growth curves from the incubation of the five different microorganisms at several concentrations of EOCs, both free and immobilised in SiO_2 and cellulose. Two types of HCL were generated: that which grouped different concentrations of EOCs (vertical clustering) and that which clustered the different microorganisms incubated in the free or immobilised EOCs (horizontal clustering). Two groups for the different concentrations of antimicrobials were observed.

Group 1 was integrated by the less effective concentrations of the different EOCs, whereas group 2 was made up mainly of the most effective concentrations, which matched the highest concentrations of all the EOCs, but also most of the thymol and eugenol concentrations. These latter two compounds displayed impaired growth at much lower concentrations. In the horizontal clustering, a clear separation was observed between the free (cluster A) and immobilised EOCs (cluster B), regardless of the tested strains and concentrations.

These growth curve data were also used to calculate the minimum inhibitory concentrations (MICs) of the different compounds (Table 1). It was noteworthy that the immobilisation of the EOCs on the two supports generally led to a major reduction in the MIC values compared with the free compounds, except for free thymol. *Aa* and *Db* obtained the highest and the lowest median MIC value, respectively, which indicated the most and least resistant microorganisms to the overall tested EOCs. The SiO_2 particles functionalised with eugenol gave the lowest MIC values for all the strains, and emerged as the most effective compound to inhibit microbial growth.

Table 1. The minimum inhibitory concentration (MIC) (mg/ml) obtained from the linear regression fit for the inhibitory effects of the different antimicrobials on the maximum growth rate (μ_{max}). Standard deviations for each parameter were obtained from three independent experiments.

	S. cerevisiae	***D. bruxellensis***	***Z. bailii***	***A. aceti***	***L. plantarum***
Thymol	0.471±0.012	0.127±0.003	0.0485±0.015	0.4945±0.071	0.116±0.002
Vanillin	1.2635±0.003	0.9305±0.017	0.527±0.024	13.235±0.298	0.6555±0.054
Eugenol	3.9785±0.101	1.4565±0.053	1.3175±0.007	20.4545±0.413	8.075±0.323
Carvacrol	2.75±0.011	0.4185±0.004	0.5255±0.052	5.625±0.201	1.1805±0.109
SiO_2	0.965±0.007	0.9055±0.025	2.3835±0.123	1.346±0.073	1.2175±0.054
SiO_2 thymol	0.781±0.002[ab]	0.558±0.002[ab]	1.314±0.021[ab]	0.8915±0.052[ab]	0.7525±0.016[ab]
SiO_2 vanillin	0.722±0.010[ab]	0.394±0.013[ab]	1.0045±0.005[ab]	0.715±0.064[ab]	1.2395±0.104[ab]
SiO_2 eugenol	0.0555±0.001[ab]	0.0405±0.002[ab]	0.05±0.011[ab]	0.2265±0.001[ab]	1.026±0.098[ab]
SiO_2 carvacrol	0.88±0.013[a]	1.1165±0.015[ab]	1.6015±0.133[ab]	0.798±0.014[ab]	0.785±0.065[ab]
Cellulose	1.345±0.053	1.97±0.103	0.7415±0.054	0.76±0.017	0.944±0.057
Cellulose thymol	0.722±0.006[ac]	0.2575±0.014[ac]	0.646±0.002[a]	0.4825±0.003[c]	0.9175±0.061[a]
Cellulose vanillin	0.528±0.009[ac]	0.7975±0.015[ac]	0.7665±0.008[a]	0.8785±0.019[ac]	1.1705±0.088[a]
Cellulose eugenol	0.1645±0.001[ac]	0.141±0.020[ac]	0.1265±0.015[ac]	0.922±0.135[a]	1.0165±0.004[a]
Cellulose carvacrol	0.766±0.014[ac]	0.2615±0.017[ac]	0.871±0.047[a]	0.76±0.107[a]	1.094±0.155

[a] Significant differences (P value ≤0.05) in each strain compared with the free molecules

[b] Significant differences (P value ≤0.05) in each strain compared with the control condition (SiO_2).

[c] Significant differences (P value ≤0.05) in each strain compared with the control condition (cellulose).

3.3. Antimicrobial activity of the EOC-functionalised membranes

As mentioned before, most wineries have filtration systems for microbial wine stabilisation. Thus we believe that transferring this technology to wineries can come for the filters functionalised with antimicrobial particles. For this purpose, we performed a filtration system with two types of cellulosic filters: (i) a cellulosic membrane functionalised with EOCs as the antimicrobial membrane; (ii) a cellulosic membrane

(0.45 µm) to hold back cells to evaluate the viability of the culture after coming into contact with the antimicrobial. The reduction in the filtered culture's viability would come immediately after microorganisms came into contact with EOCs during cellulosic membrane filtration.

The filtration of the different types of microorganisms through the EOC-functionalised membranes significantly reduced their viability. Figure 5 and Table S4 show the relative viability compared with the control of each microorganism after filtration.

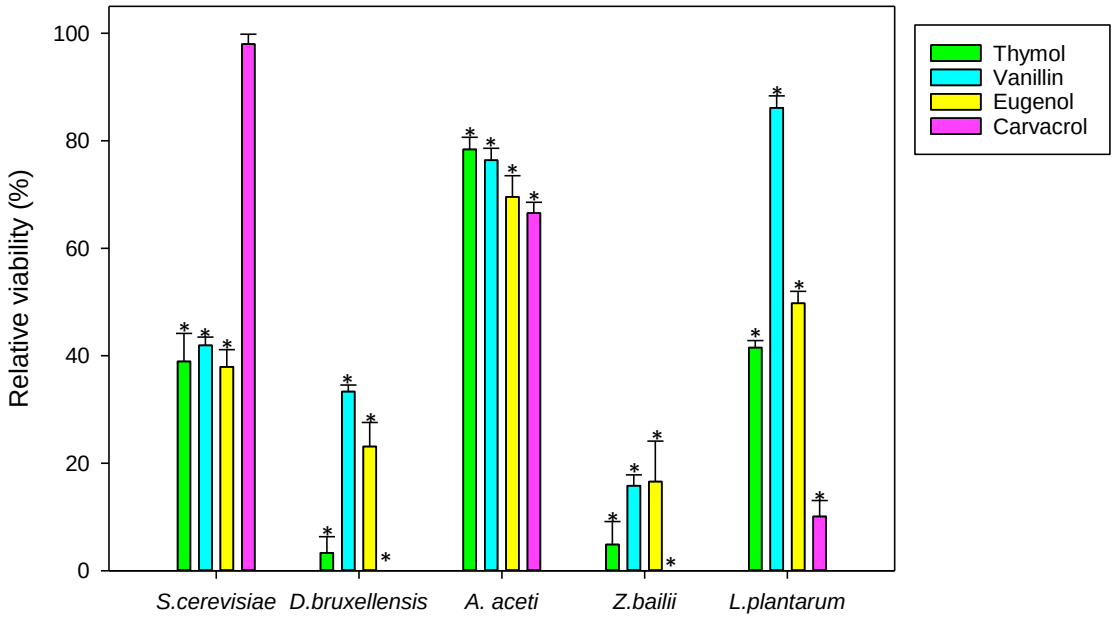

Figure 5. Relative viability (%) compared with the control (without treatment) of each microorganism after filtration through the cellulosic membranes functionalised with the different EOCs (means and standard deviations, n=3). *Significant differences compared with the control.

In this format (functionalised paper membrane), carvacrol was the most effective EOC in reducing practically 100% of viability in *Db*, *Zb* and *Lp*. However, this compound practically did not affect *Sc* viability, which was affected more by the filters functionalised with thymol and eugenol. Thymol was also most effective in reducing

viability in *Db* and *Zb*. As expected, the control condition (filtration by cellulose membrane with no antimicrobial particles) allowed 100% viability to remain (data not shown).

4. Discussion

As a result of climate change, musts have increased grape sugar concentrations that lead to high wine alcohol levels, lower acidities (higher pH) and the modification of varietal aroma compounds (Mira de Orduña, 2010). These changes in wine composition, mainly the increase in pH and the presence of residual sugars in wine, are the perfect situation for microbial spoilage. Low pH values are a cornerstone of microbiological stability. Accordingly, the trend towards higher pH values, if not corrected, poses the risk of increased microbial contamination (Mira de Orduña, 2010).

The wine industry has preserved the microbial stability of bottled wines by using chemical compounds, mainly SO_2 (Divol, Du Toit, & Duckitt, 2012; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006), and by filtering wines through sterilising filters (0.45 µm), which retains all microbial contaminants (Ribéreau-Gayon et al., 2006). However, this industry currently faces a strong reducing SO_2 trend and minimally alters the wine composition by using sterilised filtration. Thus bearing in mind the importance of microbial wine stabilisation, in the present work we propose using EOCs with antimicrobial activity immobilised in two support types. EOCs are compounds that derive from plant material with the potential to be used in natural food preservation in the future as they are natural antimicrobials (Nielsen et al., 2017). Their immobilisation in cellulose and silica supports offers the advantage of avoiding strong sensory properties, poor solubility and instability of EOCs. Moreover, the developed supports have previously shown improved antimicrobial activity against pathogen and spoilage microorganisms

compared with free compounds (Chen, Shi, Neoh, & Kang, 2009; Ribes et al., 2017; Ruiz-Rico et al., 2017).

Our results do not completely validate this statement because it was EOC-dependent. The most paradigmatic and contradictory compounds were eugenol and thymol, whose effectiveness greatly increased and decreased, respectively, when immobilised. Moreover, the effectiveness among the immobilised EOCs also depended on the type of support used; i.e., eugenol was more effective when immobilised in the silica particles, whereas thymol displayed increased effectiveness when attached to cellulose. The density of EOC on the particles' surface can determine each support's antimicrobial activity. As expected, we also observed a direct correlation between the decreased viability and impaired growth of surviving cells after the antimicrobial treatment. Likewise, the growth curves of these surviving cells allowed us to calculate the MIC values for the binomial EOC/microorganism, which correlated with the viability data.

The *in vitro* antimicrobial activity of the free EOCs has been previously described to give heterogeneous results. *Sc* was inhibited by 0.3 mg/ml of carvacrol and thymol, 1.6 mg/ml of eugenol (Mastelić et al., 2008) and 1.1 mg/ml of vanillin (Fitzgerald, Stratford, Gasson, & Narbad, 2005). Carvacrol and thymol showed *in vitro* antifungal activity against *Db* (MIC 0.03 mg/ml) at pH 6.5 (Chavan & Tupe, 2014). Those results are generally lower than the values obtained herein. Vanillin has displayed an antimicrobial effect with MIC values of 1.15 mg/ml for *Zb* (Fitzgerald et al., 2005) and 11.4 mg/ml for *Lp* (Fitzgerald et al., 2004), which are much higher MIC values than those obtained in the present work. Recently, the antifungal activity of eugenol and thymol in their free form and immobilised in mesoporous silica particles has been recently reported against *Zb*, with a minimal fungicidal concentration (MFC) of 0.4 mg/ml obtained for the free compounds and greater antimicrobial activity for eugenol after its immobilisation (MFC 0.2 mg/ml)

(Ribes et al., 2017). The enhanced antimicrobial activity of EOCs after their anchorage to a support has been stated in the microbiological assays of this work, with MIC values up to 90-fold lower than those of the free bioactive compounds (e.g. eugenol-functionalised support *vs.* free eugenol against *Aa*).

Very few examples of using alternative free or immobilised antimicrobial compounds to avoid wine spoilage can be found in the literature. Animal origin molecules like chitosan or lysozyme have been reported as antimicrobial agents for spoilage yeasts (Escudero-Abarca et al., 2004) and LAB (Gao et al., 2002). Recently, lysozyme has been covalently immobilised on spherical chitosan supports, and appeared to be more effective in the immobilised form than in the free one when applied to real white and red wine (Cappannella et al., 2016). The antifungal potential of free EOCs (carvacrol and thymol) against wine spoilage yeasts has been *in vitro* and *in situ* evaluated by Chavan & Tupe (2014). Nevertheless, immobilised EOCs have not yet been used to inactivate typical wine-spoiling microorganisms. Our results showed acetic acid bacteria *A. aceti* to be the most resistant to most of the tested EOCs and concentrations. This species has been previously described as acting as a ubiquitous bacterium throughout the winemaking process, with high tolerance to most of the antimicrobial preservatives used in oenology (Guillamón & Mas, 2017). The good news is that *D. bruxellensis*, the main nightmare for oenologists from a microbiological point of view, was very sensitive to most EOCs, and had the lowest MIC values.

In addition, the immobilisation of EOCs in cellulosic paper allowed us to test the antimicrobial effect of bioactive compounds through filtration as a proof of concept for designing a novel filter for microbial wine stabilisation. These filters should present a much larger pore size than sterilising 0.45 µm filters by much better preserving the organoleptic quality of filtered wines.

5. Conclusions

The attachment of naturally-occurring antimicrobial compounds on different supports (silica microparticles, cellulose microparticles and cellulosic paper membranes) preserved the inhibitory activity of bioactive agents against reference strains of wine spoilage. Besides preserving antimicrobial properties, the developed supports displayed enhanced inhibitory capability compared to most free bioactive compounds. The covalent immobilisation of EOCs onto the surface of different materials allowed us to obtain antimicrobial supports that can be used as processing aids in the winemaking process to prevent microbial spoilage. This new technology can fulfil consumers increasing demand for high quality fresh wines free of chemical preservatives, with an extended shelf life and produced by sustainable methods with small carbon footprints. Nevertheless, further studies are needed to establish the mechanism of action of the immobilised bioactive compounds and to evaluate their efficacy in real wine matrices.

Acknowledgements

Authors gratefully acknowledge the financial support from the Ministerio de Economía y Competitividad and FEDER-EU (Projects AGL2015-70235-C2-1-R and AGL2016-77505-C3-1-R, granted to JMB and JMG, respectively). The authors also thank the Electron Microscopy Service at the UPV for support. Authors thank Antonio Ruiz for technical assistance.

References

Aguilera, J., Randez-Gil, F., & Prieto, J. A. (2007). Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. *FEMS Microbiology Reviews*, *31*(3), 327–41. https://doi.org/10.1111/j.1574-6976.2007.00066.x

Andorrà, I., Landi, S., Mas, A., Guillamón, J., & Esteve-Zarzoso, B. (2008). Effect of oenological practices on microbial populations using culture- independent techniques. *Food Microbiology*, *25*, 849–856. https://doi.org/10.1016/j.fm.2008.05.005

Arriagada-Carrazana, J. P., Sáez-Navarrete, C., & Bordeu, E. (2005). Membrane filtration effects on aromatic and phenolic quality of Cabernet Sauvignon wines. *Journal of Food Engineering*, *68*(3), 363–368. https://doi.org/10.1016/J.JFOODENG.2004.06.011

Bartowsky, E. J., Xia, D., Gibson, R. L., Fleet, G. H., & Henschke, P. A. (2003). Spoilage of bottled red wine by acetic acid bacteria. *Letters in Applied Microbiology*, *36*, 307–314. https://doi.org/10.1046/j.1472-765X.2003.01314.x

Bernardos, A., Marina, T., Žáček, P., Pérez-Esteve, É., Martínez-Mañez, R., Lhotka, M., … Klouček, P. (2015). Antifungal effect of essential oil components against *Aspergillus niger* when loaded into silica mesoporous supports. *Journal of the Science of Food and Agriculture*, *95*(14), 2824–2831. https://doi.org/10.1002/jsfa.7022

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*(3), 223–253. https://doi.org/10.1016/J.IJFOODMICRO.2004.03.022

Cappannella, E., Benucci, I., Lombardelli, C., Liburdi, K., Bavaro, T., & Esti, M. (2016). Immobilized lysozyme for the continuous lysis of lactic bacteria in wine: Bench-scale fluidized-bed reactor study. *Food Chemistry*, *210*, 49–55. https://doi.org/10.1016/J.FOODCHEM.2016.04.089

Chavan, P. S., & Tupe, S. G. (2014). Antifungal activity and mechanism of action of carvacrol and thymol against vineyard and wine spoilage yeasts. *Food Control*, *46*,

115–120. https://doi.org/10.1016/J.FOODCONT.2014.05.007

Chen, F., Shi, Z., Neoh, K. G., & Kang, E. T. (2009). Antioxidant and antibacterial activities of eugenol and carvacrol-grafted chitosan nanoparticles. *Biotechnology and Bioengineering*, *104*(1), 30–39. https://doi.org/10.1002/bit.22363

Divol, B., Du Toit, M., & Duckitt, E. (2012). Surviving in the presence of sulphur dioxide: Strategies developed by wine yeasts. *Applied Microbiology and Biotechnology*, *95*(3), 601–613. https://doi.org/10.1007/s00253-012-4186-x

Du Toit, M., & Pretorius, I. S. (2000). Microbial spoilage and preservation of wine: using weapons from nature's own arsenal-A review. *South African Journal for Enology and Viticulture*, *21*, 74–96. Retrieved from http://www.sawislibrary.co.za/dbtextimages/dutoitm1.pdf

Dunn, L. L., Davidson, P. M., & Critzer, F. J. (2016). Antimicrobial efficacy of an array of essential oils against lactic acid bacteria. *Journal of Food Science*, *81*(2), M438–M444. https://doi.org/10.1111/1750-3841.13181

Escudero-Abarca, B. I., Aguilar-Uscanga, M. G., Hayward-Jones, P. M., Mendoza, P., Ramírez, M., & Gómez-Rivas, L. (2004). Selective antimicrobial action of chitosan against spoilage yeasts in mixed culture fermentations. *Journal of Industrial Microbiology and Biotechnology*, *31*(1), 16–22. https://doi.org/10.1007/s10295-004-0112-2

Fitzgerald, D. J., Stratford, M., Gasson, M. J., & Narbad, A. (2005). Structure−function analysis of the vanillin molecule and its antifungal properties. *Journal of Agricultural and Food Chemistry*, *53*(5), 1769–1775. https://doi.org/10.1021/JF048575T

Fitzgerald, D. J., Stratford, M., Gasson, M. J., Ueckert, J., Bos, A., & Narbad, A. (2004). Mode of antimicrobial action of vanillin against *Escherichia coli*,

Lactobacillus plantarum and *Listeria innocua*. *Journal of Applied Microbiology*, *97*(1), 104–113. https://doi.org/10.1111/j.1365-2672.2004.02275.x

Fleet, G. H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, *86*(1–2), 11–22. https://doi.org/10.1016/S0168-1605(03)00245-9

Gao, Y. C., Zhang, G., Krentz, S., Darius, S., Power, J., & Lagarde, G. (2002). Inhibition of spoilage lactic acid bacteria by lysozyme during wine alcoholic fermentation. *Australian Journal of Grape and Wine Research*, *8*(1), 76–83. https://doi.org/10.1111/j.1755-0238.2002.tb00214.x

García-Ríos, E., Gutiérrez, A., Salvadó, Z., Arroyo-López, F. N., & Guillamon, J. M. (2014). The fitness advantage of commercial wine yeasts in relation to the nitrogen concentration, temperature, and ethanol content under microvinification conditions. *Applied and Environmental Microbiology*, *80*(2), 704–713. https://doi.org/10.1128/AEM.03405-13

Gill, A. O., & Holley, R. A. (2006). Disruption of *Escherichia coli, Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *International Journal of Food Microbiology*, *108*(1), 1–9. https://doi.org/10.1016/j.ijfoodmicro.2005.10.009

Guillamón, J. M., & Mas, A. (2017). Acetic acid bacteria. In *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 43–64). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-60021-5_2

Hyldgaard, M., Mygind, T., & Meyer, R. L. (2012). Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. *Frontiers in Microbiology*, *3*, 1–24. https://doi.org/10.3389/fmicb.2012.00012

Kuanyshev, N., Adamo, G., Porro, D., & Branduardi, P. (2017). The spoilage yeast *Zygosaccharomyces bailii*: Foe or friend? *Yeast (Chichester, England)*, *34*, 359–

370. https://doi.org/10.1002/yea

Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek*, *76*(1–4), 317–31.

Mastelić, J., Jerković, I., Blažević, I., Poljak-Blaži, M., Borović, S., Ivančić-Baće, I., … Müller, N. (2008). Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. *Journal of Agricultural and Food Chemistry*, *56*(11), 3989–3996. https://doi.org/10.1021/jf073272v

Mira de Orduña, R. (2010). Climate change associated effects on grape and wine quality and production. *Food Research International*, *43*(7), 1844–1855. https://doi.org/10.1016/j.foodres.2010.05.001

Nielsen, C. K., Kjems, J., Mygind, T., Snabe, T., Schwarz, K., Serfert, Y., & Meyer, R. L. (2017). Antimicrobial effect of emulsion-encapsulated isoeugenol against biofilms of food pathogens and spoilage bacteria. *International Journal of Food Microbiology*, *242*, 7–12. https://doi.org/10.1016/J.IJFOODMICRO.2016.11.002

Petrova, B., Cartwright, Z., & Edwards, C. (2016). Effectiveness of chitosan preparations against *Brettanomyces bruxellensis* grown in culture media and red wines. *Oeno One*, *50*, 49–56.

Prakash, B., Kedia, A., Mishra, P. K., & Dubey, N. K. (2015). Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities – Potentials and challenges. *Food Control*, *47*, 381–391. https://doi.org/10.1016/J.FOODCONT.2014.07.023

Ribéreau-Gayon, P., Glories, Y., Maujean, A., & Dubourdieu, D. (2006). *Handbook of Enology, The Chemistry of Wine: Stabilization and Treatments: Second Edition*. *Handbook of Enology, The Chemistry of Wine: Stabilization and Treatments: Second Edition* (Vol. 2). wiley.

Ribes, S., Ruiz-Rico, M., Pérez-Esteve, É., Fuentes, A., Talens, P., Martínez-Máñez, R., & Barat, J. M. (2017). Eugenol and thymol immobilised on mesoporous silica-based material as an innovative antifungal system: Application in strawberry jam. *Food Control*, *81*. https://doi.org/10.1016/j.foodcont.2017.06.006

Ruiz-Rico, M., Fuentes, C., Pérez-Esteve, T., Jiménez-Belenguer, A. I., Quiles, A., Marcos, M. D., … Barat, J. M. (2015). Bactericidal activity of caprylic acid entrapped in mesoporous silica nanoparticles. *Food Control*, *56*. https://doi.org/10.1016/j.foodcont.2015.03.016

Ruiz-Rico, M., Pérez-Esteve, É., Bernardos, A., Sancenón, F., Martínez-Máñez, R., Marcos, M. D., & Barat, J. M. (2017). Enhanced antimicrobial activity of essential oil components immobilized on silica particles. *Food Chemistry*, *233*. https://doi.org/10.1016/j.foodchem.2017.04.118

Rupasinghe, H. P. V., Boulter-Bitzer, J., Ahn, T., & Odumeru, J. A. (2006). Vanillin inhibits pathogenic and spoilage microorganisms in vitro and aerobic microbial growth in fresh-cut apples. *Food Research International*, *39*(5), 575–580. https://doi.org/10.1016/J.FOODRES.2005.11.005

Salvadó, Z., Arroyo-López, F. N., Barrio, E., Querol, A., & Guillamón, J. M. (2011). Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*. *Food Microbiology*, *28*(6), 1155–1161. https://doi.org/10.1016/j.fm.2011.03.008

Shah, B., Davidson, P. M., & Zhong, Q. (2012). Nanocapsular dispersion of thymol for enhanced dispersibility and increased antimicrobial effectiveness against *Escherichia coli* O157:H7 and *Listeria monocytogenes* in model food systems. *Applied and Environmental Microbiology*, *78*(23), 8448–53. https://doi.org/10.1128/AEM.02225-12

Smith, B. D., & Divol, B. (2016). *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages. *Food Microbiology*, *59*, 161–175. https://doi.org/10.1016/j.fm.2016.06.008

Sow, L. C., Tirtawinata, F., Yang, H., Shao, Q., & Wang, S. (2017). Carvacrol nanoemulsion combined with acid electrolysed water to inactivate bacteria, yeast in vitro and native microflora on shredded cabbages. *Food Control*, *76*, 88–95. https://doi.org/10.1016/j.foodcont.2017.01.007

Turek, C., & Stintzing, F. C. (2013). Stability of essential oils: A review. *Comprehensive Reviews in Food Science and Food Safety*, *12*(1), 40–53. https://doi.org/10.1111/1541-4337.12006

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., & Van, K. (1990). Modeling of the bacterial growth curve modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, *56*(6), 1875–1881.