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Microbial stabilization of craft beer by filtration through silica supports functionalized with essential oil components

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Abstract

The brewing industry uses conventional pasteurization to assure beer microbial stability, but this process compromises its quality characteristics. This study proposes a novel cold pasteurization technology based on filtration through silica microparticles (5, 10, 25 or 50 µm) functionalized with essential oil components (EOCs). After the synthesis and characterization of the supports, craft beer was filtered through a bed of EOC-functionalized particles to assess their capability to entrap and/or inactivate beer microbiota. The microbiological analysis of filtered beer showed that the supports presented remarkable removal capacity against *Escherichia coli*, mesophilic bacteria, lactic acid bacteria, and mold and yeast. The preservation potential of the filtration technology remained steady after filtering multiple samples and previous washing with a high water volume. The determination of potential leaching of the immobilized EOCs resulted in zero release of the grafted molecules in the beer samples filtered through the bed of particles. Moreover, differences among control and filtered beers detected by a panel of untrained judges were scarce or nonexistent. The proposed technology can be considered an effective novel mild preservation method for craft beer as it can reduce the microbial load of the product and can prevent negative effects on the sensory properties of beverages.

Keywords: cold pasteurization; immobilization; naturally-occurring antimicrobials; spoilage microorganism; beverages.

1. Introduction

Beer is considered a microbiologically safe beverage given the presence of ethanol, carbon dioxide, low concentrations of nutritive substances, low pH, and low oxygen content (Sakamoto & Konings, 2003). However, some spoilage microorganisms can grow and may shorten beer shelf life (Cao, Zhou, Guo, & Li, 2011). Lactic acid bacteria (LAB) are the most detrimental Gram-positive bacteria for beer as they produce organoleptic changes that can cause high economic loss (Lu et al., 2010). The main Gram-negative bacteria responsible for beer spoilage are anaerobic bacteria of genera *Pectinatus* and *Megasphaera* (Sakamoto & Konings, 2003). Spoilage can also be produced by wild yeasts that may result in serious problems because of the difficulty to distinguish them from brewing yeasts (Vaughan, O'Sullivan, & Sinderen, 2005).

The brewing industry prevents microbial contamination by thermal pasteurization, which can provoke alterations to the organoleptic properties. Pasteurization increases the level of oxidation in beer, which results in loss of antioxidants, and also in changes in amino acids and proteins (Lund, Hoff, Berner, Lametsch, & Andersen, 2012). When exposed to heat, beer undergoes color and flavor changes (Cao et al., 2011; Franchi, Tribst, & Cristianini, 2011), as well as structural modifications by lower foam stability and higher turbidity because of the formation of new tannin-protein complexes with denaturized proteins (Deng et al., 2018).

Thus interest in non-thermal treatments, including high-pressure processing (Milani, Ramsey, & Silva, 2016), pulsed electric fields (Walkling-Ribeiro, Rodríguez-González, Jayaram, & Griffiths, 2011), ultraviolet irradiation (Lu et al., 2010) or ultrasound (Deng et al., 2018), has risen to extend beer shelf life. However, they have some implementation issues, such as limited efficacy, changes in food properties and high investment and production costs, which have limited their industrial application (Morris, Brody, & Wicker, 2007; Walkling-Ribeiro et al., 2011; Yang, Huang, Lyu, & Wang, 2016).

Filtration is an important process for the beverage industry, which is used to stabilize, clarify and/or concentrate liquids through the removal of solid particles (Fuenmayor, Lemma, Mannino, Mimmo, & Scampicchio, 2014). Cold-sterilization of beer by filtration through filters with a pore diameter less than 0.2 μm provokes high retention of essential compounds, obtaining an insipid product with significant loss of color, dry matter, bitterness and foam. Besides, fouling and cleaning requirements are critical factors for the extensive application of this technology (Fillaudeau & Carrère, 2002). Otherwise, filtration based

on sand or diatomaceous earth is used to remove organic matter and microorganisms from liquids using bigger pore size, but these do not fulfill the efficiency needed when removing pathogens and present regeneration/disposal issues (Devi, Alemayehu, Singh, Kumar, & Mengistie, 2008).

A new antimicrobial system, based on the covalent immobilization of naturally-occurring antimicrobial molecules on supports, has been recently developed by our research group. This invention involves the grafting of bioactive compounds to the surface of silica particles by preserving and enhancing their antimicrobial effect (Ribes et al., 2017; Ruiz-Rico et al., 2017). The present study proposes the application of these novel antimicrobial supports as filtering materials for the cold pasteurization of craft beer. Thus the objective is to develop supports based on essential oil components (EOCs) immobilized onto silica microparticles, and to assess the filtration potential against beer microbiota.

2. Material and methods

2.1 Reagents

Carvacrol, eugenol, thymol, trimethylamine, (3-aminoproppyl)triethoxysilane (APTES), 2-butanone, chloroform, paraformaldehyde, sodium borohydride, KOH and silica particles (10, 25 and 50 µm) were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile, methanol, diethyl ether, dichloromethane, NaCl, $MgSO_4$, H_2SO_4 and HCl 37% were obtained from Scharlab (Barcelona, Spain). Vanillin was purchased from Ventós (Barcelona, Spain). Silica particles (SYLYSIA® SY350/FCP, 5 µm) were acquired from Silysiamont (Milano, Italy). Plate Count Agar (PCA), Man, Rogosa and Sharpe agar (MRS), Tryptic Soy Broth (TSB) and Peptone Dextrose Agar (PDA) were supplied by Scharlab (Barcelona, Spain). Pasteurized commercial beer (Pale Ale, 5.0% v/v) and unpasteurized commercial craft beer (American Pale Ale, 5.6% v/v), purchased in a local supermarket, were used to assess the effectiveness of the filtration system as a preservation treatment.

2.2 Preparing the antimicrobial supports

The EOC-functionalized particles were synthesized according to the methodology described by Ruiz-Rico et al. (2017), with some modifications. First, the aldehyde derivatives of carvacrol and thymol were synthesized by direct formylation, and eugenol aldehyde was prepared using a Reimer–Tiemann reaction. The aldehydes of carvacrol, eugenol and thymol and pure vanillin were reacted with APTES to obtain the

corresponding alkoxysilane derivatives. Then these derivatives were immobilized on the surface of silica particles with different mean sizes (5, 10, 25 or 50 μm). Afterward, the reduction of the imine bond formed between the aldehyde moiety of the bioactive compounds and the amine group of APTES was carried out to optimize anchorage in presence of sodium borohydride.

2.3 Antimicrobial supports characterization

The particles' morphology was characterized by field emission scanning electron microscopy under a Zeiss Ultra 55 microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany), observed in the secondary electron mode. The zeta potential analysis was performed in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) after the sonication of particle suspensions in water. The degree of functionalization was determined by thermo-gravimetric analyses (TGA) and elemental analyses. TGA determinations were made on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc., Schwarzenbach, Switzerland) with a heating program that consisted of a heating ramp of 10 °C/min from 25 to 800 °C in an oxidant atmosphere (air, 80 mL/min).

2.4 Beer filtration

The effect of filtration with the 20 particles prepared in section 2.2 on the microbiological and sensory properties of filtered beers was evaluated by different filtration tests as follows:

Test 1: Studying the retention capability after filtering 100 mL of beer through the bed of particles.

Test 2: Studying the influence of previous washing with a high water volume, as a pre-conditioning treatment, on the retention properties. To this end, 1 L of sterile water was filtered through the particles prior to filtering 100 mL of craft beer.

Test 3: Assessing the reuse capability of the supports by filtering three consecutive samples of 100 mL of craft beer.

Test 4: Studying the combined effect of washing and filtering multiple samples on the removal capacity. The particles were washed with 1 L of sterile water as a previous treatment for the filtration of all three samples.

These assays were performed using a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany) connected to a sterile Erlenmeyer flask to collect the sample. In all the cases, a bed

of different silica microparticles (thickness of 0.5 cm) was used. Each test was carried out in triplicate, using different batches of beer to include natural variability in the beer preparation. Two control samples, these being non-filtered beer and beer filtered through a bed of non-functionalized silica particles, were included.

The effectiveness of the supports as filtering materials was first tested with beer inoculated with *Escherichia coli*. This coliform microorganism was selected due to the potential occurrence of food-borne illnesses associated with beer contamination (Lu et al., 2010). The assay was performed using pasteurized beer inoculated with *E. coli* K12 (CECT 433, Colección Española de Cultivos Tipo, Spain) as surrogate of pathogenic *E. coli* strains.

To prepare the inoculum, a colony was transferred to a test tube with 10 mL of TSB and incubated at 37 °C for 24 h. The inoculum was centrifuged at 4000 rpm for 10 min and the precipitated cells were resuspended in 1 L of beer to obtain a microbial density of 10^6-10^7 cells/mL. The inoculated beer was filtered according to *Test 1*. The count values after plating and incubation of collected beer in PCA (37 °C, 24 h) were logarithmically transformed and expressed as $\log_{10}$ CFU/mL, with a limit of detection of 5 CFU/mL.

To check the effectiveness of filtration to stabilize the microorganisms naturally present in craft beer, samples collected after *Tests 1-4* were plated in different media. For the enumeration of aerobic mesophilic bacteria, beer was plated in-depth in PCA and plates were incubated at 30 °C for 72 h. LAB were counted after plating samples in-depth in MRS agar and incubation at 37 °C for 48 h. For the enumeration of mold and yeast, beer was plated on surfaces in PDA and plates were incubated at 25 °C for 72 h.

2.5 EOCs leaching

The potential leaching of the immobilized EOCs was evaluated after filtering 100 mL of beer through pre-conditioned filter (*Test 2*). The quantification of the compounds in the beer was carried out by extraction using QuEChERS procedure followed by gas chromatography-mass spectrometry (GC-MS).

The QuEChERS procedure combine two stages, analytes extraction with an organic solvent and different salts and clean-up of the organic extract by dispersive solid-phase extraction. The use of a clean-up step

was needed to avoid the deleterious effect of several matrix components (Valente, Santos, Moreira, & Rodrigues, 2013).

The analysis was performed in a 6890/5975 inert GC-MS (Agilent Technologies, USA), equipped with a HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The oven temperature was held at 60 °C for 3 min, and then raised to 100 °C at 10 °C/min, to 140 °C at 5 °C/min, and finally to 240 °C at 20 °C/min. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperatures were set at 250 °C and 230 °C, respectively. EOCs were quantified according to the external standard method, in which a calibration curve of the peak area was used against the compound concentration. Besides the amount of released EOCs, the percentage of leached compounds was calculated considering the EOCs attached to the supports and the amount of particles needed to prepare the bed of particles.

2.6 Sensory evaluation

A sensory analysis was performed to evaluate the acceptance of the beer filtered through the most suitable EOC-functionalized supports, according to the results obtained in the filtration assays and the leaching determination. Non-filtered craft beer was also included in the analysis.

The panel involved 51 non-trained panelists (28 females, 23 males), whose ages ranged from 23 to 62 years. Tests were conducted using a 9-point hedonic scale (1 = dislike very much, 9 = like very much). Four sensory parameters were evaluated (appearance, color, odor and general acceptance) and each coded sample was served to the panelists at room temperature in a capped transparent glass vial.

2.7 Statistical analysis

Data were statistically analyzed with Statgraphics Centurion XVI (Statpoint Technologies Inc., Warrenton, USA). Results obtained in filtration assays were evaluated by a multifactor analysis of variance to establish the effect of immobilized bioactive compound, particle size and number of filtrations. Data obtained in the characterization of the antimicrobial supports and sensory analysis were analyzed by a one-way ANOVA to discriminate among samples. The least significant difference procedure was used to test the differences between averages at the 5% significance level.

3. Results

3.1 Characterization of antimicrobial supports

Figure 1 shows the morphology of non-modified and carvacrol-functionalized supports, used as reference particles given the similarity between the different functionalized particles. The smallest particles (5 µm) have a sphere-like shape and an irregular surface. In contrast, the silica particles of sizes 10-50 µm have an irregular prism shape and a smooth surface. The supports present a homogeneous particle size rate, except for the 25 µm-particles that have a wider size range in accordance with the technical information of the specification sheet that establishes a particle size that falls within the 5-25 µm range. In all cases, functionalization did not affect the structure of the silica microparticles.

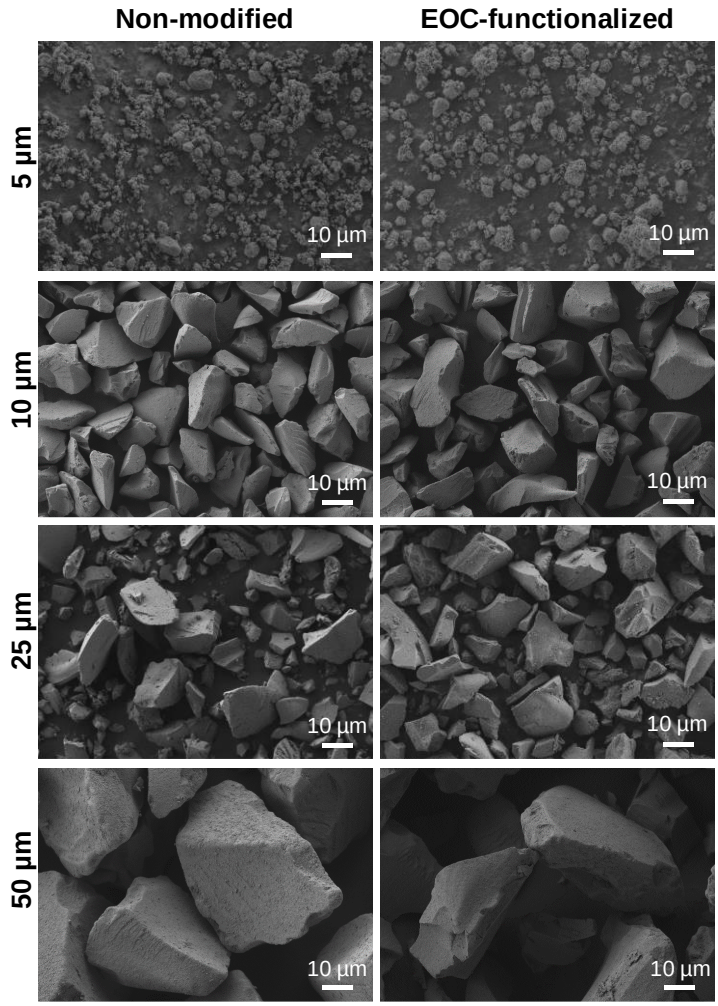

Figure 1. Field emission scanning electron microscopy images of the bare and carvacrol-functionalized silica microparticles.

Table 1 presents the zeta potential of the supports. Bare particles exhibited negative zeta potential because of silanol moieties. In contrast, EOC-functionalized supports presented positive zeta potential values due to the attachment of alkoxysilane derivatives. The results of the degree of functionalization showed that EOC-functionalized supports had an amount of attached bioactive compounds of ca. 0.01 g EOC/g SiO_2 for carvacrol and thymol, 0.04 g EOC/g SiO_2 for eugenol and 0.12 g EOC/g SiO_2 for vanillin on the different supports.

Table 1. Zeta potential values (mV) of the bare and EOC-functionalized silica microparticles. Mean values ± SD (n=3).

Size	***Bare***	***Carvacrol***	***Eugenol***	***Thymol***	***Vanillin***	***α***
5 µm	-29.4 ± 1.2^{a}	30.0 ± 0.6^{d}	11.7 ± 0.7^{b}	25.0 ± 1.9^{c}	29.2 ± 0.7^{d}	***
10 µm	-24.4 ± 3.0^{a}	8.0 ± 2.4^{b}	12.5 ± 5.8^{bc}	13.9 ± 1.6^{c}	23.0 ± 1.5^{d}	***
25 µm	-33.3 ± 1.0^{a}	2.3 ± 0.9^{b}	41.9 ± 2.1^{e}	12.5 ± 2.9^{c}	23.4 ± 1.1^{d}	***
50 µm	-17.1 ± 4.9^{a}	6.6 ± 2.3^{b}	30.9 ± 5.6^{c}	21.0 ± 10.2^{c}	24.9 ± 1.3^{c}	***

Same letters in the same row indicate homogeneous group membership. ***p<0.001

3.2 Effect of filtration on *E. coli* reduction

Figure 2 shows the *E. coli* counts after filtering inoculated beer through the non-functionalized and EOC-functionalized supports. The use of non-functionalized supports resulted in a slight 1-log reduction. In contrast, the filtration with the EOC-functionalized particles reduced *E. coli* in beer from approximately 10^7 CFU/mL to below 10^3 CFU/mL in most of the samples, and even to non-detectable limits for the 25 µm-support functionalized with carvacrol. The supports with mean size of 10 and 25 µm were the most effective filtering materials for reducing *E. coli*.

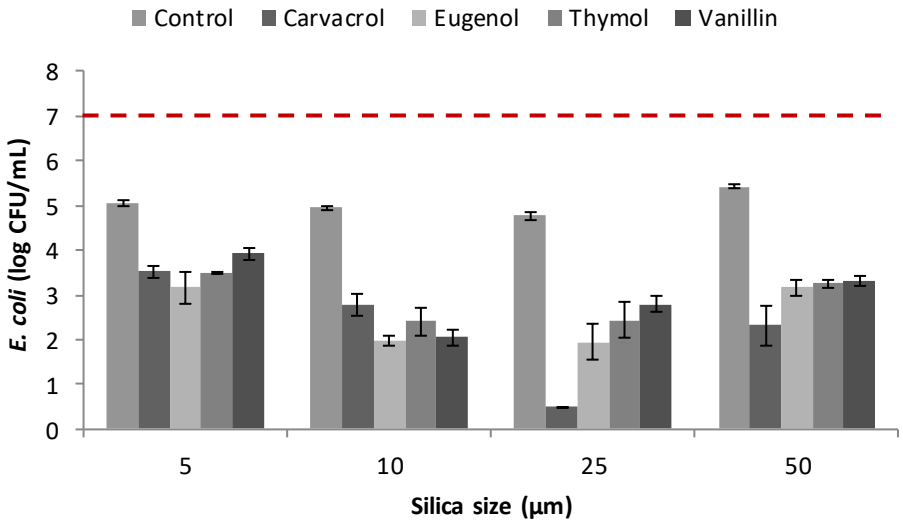

Figure 2. Microbial counts (log CFU/mL) of *E. coli* inoculated in commercial pasteurized beer after filtering beer with non-functionalized particles (control) and EOC-functionalized supports. Mean values ± SD (n=3).

3.3. Effect of filtration on beer microbiota reduction

The removal capability of the supports was also assessed against naturally contaminating microorganisms of unpasteurized craft beer. The microbial counts from the unfiltered beer were 4.67±0.33, 4.34±0.05 and 4.47±0.33 log CFU/mL for mesophilic, LAB and mold and yeast, respectively.

Figure 3 shows the counts of beer microbiota after filtering beer through non-functionalized and EOC-functionalized supports. Filtering beer through bare particles slightly lowered the microbial counts, mainly for the 25 µm-support. This material had a mean particle size that fell within the 5-25 µm range, which may favor the entrapment of microbial cells on the bed because particles of diverse size range formed a more homogeneous particle layer with smaller holes. The microbial reduction after filtration with non-modified supports ranged between 0.3-0.9 logarithmic cycles for the different supports, which confirms the very limited removal capability of bare particles. For this reason, it would be necessary to use the antimicrobial compounds anchored to the supports.

As can be seen in Figure 3, a clear reduction in the microbial load of beer filtered through any of the functionalized supports was achieved. The statistical analysis showed the significant influence of immobilized EOC and, especially, particle size, as well as their interaction on microbial reduction ($p<0.05$). The 10-25 µm functionalized particles were the most effective materials, and completely inhibited the

microorganisms with some exception. In contrast, the 5 µm particles displayed heterogeneous results with microbial reduction falling within 40-100% for the spoilage microorganisms.

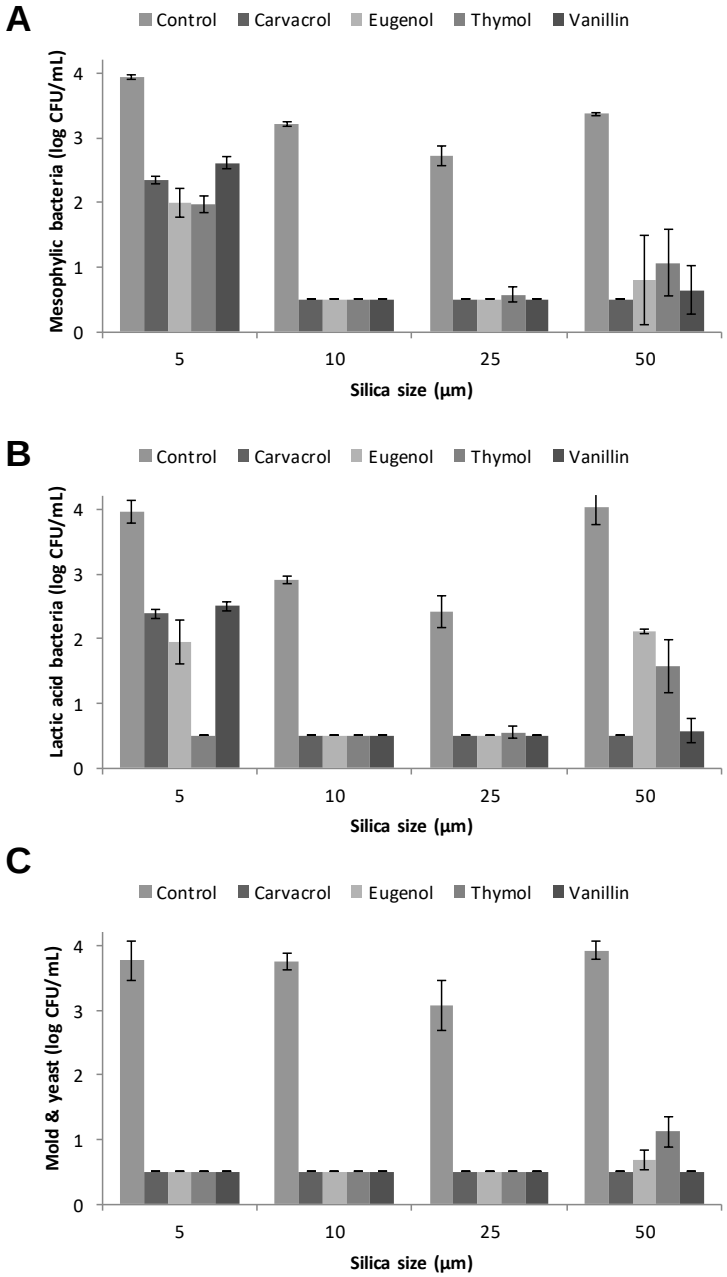

Figure 3. Microbial counts (log CFU/mL) of mesophilic bacteria (A), lactic acid bacteria (B) and mold and yeast (C) after filtering beer with non-functionalized particles (control) and EOC-functionalized supports. Mean values ± SD (n=3).

3.4. Effect of pre-conditioning on the retention properties

Figure 4 shows the microbial counts of beer after filtration through the particles previously washed with water. The results revealed that pre-conditioning generally improved the removal capability of the functionalized materials. The statistical analysis confirms the influence of the immobilized EOC and particle size on mesophilic and LAB counts ($p<0.01$).

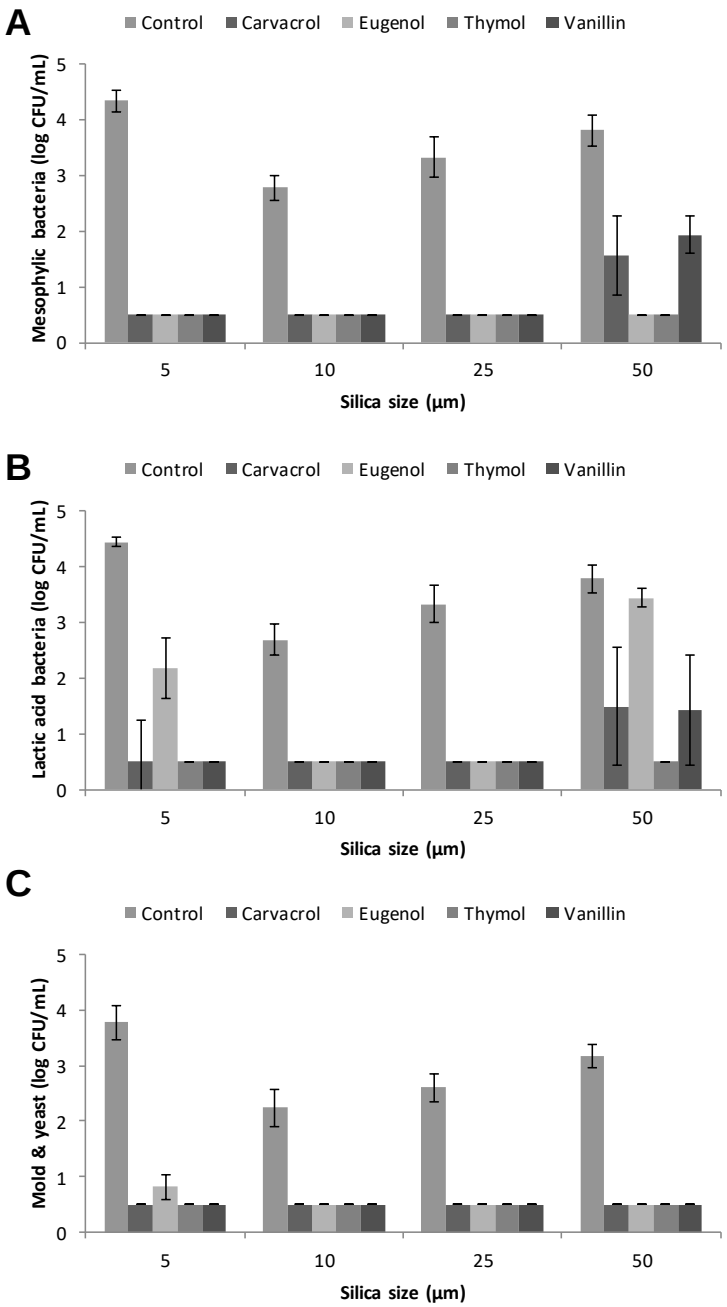

Figure 4. Microbial counts (log CFU/mL) of mesophilic bacteria (A), lactic acid bacteria (B) and mold and yeast (C) after pre-washing and filtering beer with non-functionalized particles (control) and EOC-functionalized supports. Mean values ± SD (n=3).

The retention capacity of the 5 µm-supports greatly improved after pre-conditioning. For mold and yeast, the filtration reduced counts in beer from 10^4 CFU/mL to undetectable limits. The functionalized particles of 10-25 µm were the most effective materials, and thymol was the bioactive compound that produced the most marked microbial reduction.

3.5. Evaluating the reusability of the filtering materials

Table 2 shows the microbial counts after filtering three beer samples. The retention properties of the supports of 10-25 µm remained after filtering multiple samples. The removal capacity was enhanced after filtering the consecutive samples for the 5-µm supports. In contrast, the 50-µm supports showed heterogeneous results according to the target microorganism and the immobilized EOC. The statistical analysis of the results confirmed the influence of particle size, immobilized EOC, the interaction between them, and the interaction between particle and number of samples on the microbial reduction of beer microbiota ($p<0.05$).

Table 2. Microbial counts (log CFU/mL) after washing with sterile water and filtering three samples through EOC-functionalized supports. Mean values ± SD (n=3). N: number of filtered samples.

		Mesophilic bacteria				**Lactic acid bacteria**				**Mold and yeast**			
	N	**Carvacrol**	**Eugenol**	**Thymol**	**Vanillin**	**Carvacrol**	**Eugenol**	**Thymol**	**Vanillin**	**Carvacrol**	**Eugenol**	**Thymol**	**Vanillin**
	1	2.6 ± 0.4	1.8 ± 0.1	nd	2.4 ±0. 4	2.4 ± 0.3	1.6 ± 0,2	nd	2.3 ± 0.1	nd	nd	nd	nd
5 µm	2	1.6 ± 0.4	1.0 ± 0.2	nd	1.8 ± 0.6	1.5 ± 0.3	1.1 ± 0.1	nd	2.6 ± 0.3	nd	nd	nd	nd
	3	0.3 ± 0.1	nd	nd	0.5 ± 0.1	1.1± 0.1	0.6 ± 0.1	nd	1.1 ± 0.2	nd	nd	nd	nd
	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10 µm	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1	nd	nd	0.6 ± 0.2	nd	nd	nd	0.6 ± 0.2	nd	nd	nd	nd	nd
25 µm	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1	nd	1.9 ± 0.0	1.1 ± 0.7	0.7 ± 0.2	nd	2.3 ± 0.7	1.4 ± 0.8	1.0 ± 0.1	nd	1.0 ± 0.5	1.0 ± 0.4	nd
50 µm	2	0.8 ± 0.1	2.1± 0.2	1.0 ± 0.3	0.7± 0.3	0.8 ± 0.3	2.6 ± 0.6	1.7 ± 0.9	nd	nd	1.3 ± 0.7	nd	nd
	3	1.9 ± 0.2	2.2 ± 0.6	2.0 ± 0.3	nd	2.5± 0.9	2.9 ± 0.2	2.5 ± 0.1	nd	0.9 ± 0.6	2.3 ± 0.2	1.0 ± 0.2	nd

nd (no detected, <5 CFU/mL)

Likewise, Table 3 presents the microbial load after filtering multiple samples and interspersing the previous washing of the supports with water. The pre-conditioning improved the retention efficacy of the

supports, in accordance with the results described in Figure 3. Therefore, washing with a high water volume (3 L in all) preserved the removal properties.

Table 3. Microbial counts (log CFU/mL) after washing with sterile water and filtering three samples through EOC-functionalized supports. Mean values ± SD (n=3). N: number of filtered samples.

		Mesophilic bacteria				Lactic acid bacteria				Mold and yeast			
	N	**Carvacrol**	**Eugenol**	**Thymol**	**Vanillin**	**Carvacrol**	**Eugenol**	**Thymol**	**Vanillin**	**Carvacrol**	**Eugenol**	**Thymol**	**Vanillin**
	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5 µm	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10 µm	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.3 ± 0.3	nd
	1	nd	nd	nd	nd	nd	nd	nd	0.6 ± 0.1	nd	nd	nd	nd
25 µm	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1	1.6 ± 0.6	nd	nd	1.1 ± 0.6	nd	0.9 ± 0.2	nd	nd	nd	nd	nd	nd
50 µm	2	2.5 ± 0.1	1.0 ± 0.2	1.3 ± 0.7	2.4 ± 0.2	1.2 ± 0.6	1.0 ± 0.3	1.0 ± 0.3	2.1 ± 0.2	nd	nd	nd	nd
	3	3.0 ± 0.2	1.1 ± 0.4	1.3 ± 0.8	2.7 ± 0.2	2.6 ± 0.2	1.0 ± 0.6	1.0 ± 0.3	2.5 ± 0.3	nd	nd	nd	nd

nd (no detected, <5 CFU/mL)

3.6. EOCs leaching

Table S1 presents the amount of lixiviated EOCs and the percentage of leached compounds in the effluent after filtering 100 mL beer through a pre-conditioned bed of particles (*Test 2*). As can be observed, zero release was observed in all the cases.

3.7. Sensory evaluation

Table 4 shows the scores of the evaluated attributes for non-filtered and filtered beer through the EOC-functionalized supports of 10-25 µm following the *Test 2*. The statistical analysis showed that the size of support did not significantly influence the results, and then the sensory evaluation was analyzed to establish the influence of immobilized EOC on the beer attributes. As can be seen in Table 4, the non-filtered beer received the highest scores in each of the evaluated attributes. Beer filtered through the EOC-functionalized supports resulted in minimal score differences. Only the beers filtered through the

vanillin-functionalized particles decreased more than a point in some attributes, with the respect to the control sample in a 1-9 scale.

Table 4. Average score of the different attributes evaluated in non-filtered and filtered beer. Mean values ± SD (n=51).

Sample	Apperance	Color	Odor	Acceptance
Non-filtered	7.1 ± 1.5^{a}	7.3 ± 1.4^{a}	7.2 ± 1.5^{a}	7.2 ± 1.3^{a}
Carvacrol	6.9 ± 1.3^{a}	7.0 ± 1.3^{ab}	6.2 ± 1.6^{bc}	6.4 ± 1.5^{b}
Eugenol	6.6 ± 1.5^{a}	6.7 ± 1.4^{b}	6.4 ± 1.6^{b}	6.4 ± 1.5^{b}
Thymol	6.9 ± 1.2^{a}	7.0 ± 1.3^{ab}	6.7 ± 1.5^{ab}	6.7 ± 1.4^{ab}
Vanillin	5.4 ± 1.6^{b}	5.1 ± 1.7^{c}	5.8 ± 1.7^{c}	5.5 ± 1.7^{c}

Same letters in a column indicate homogeneous group membership. ***p<0.001

4. Discussion

Conventional filtration in the beer industry are based on microfiltration using filter aids with a small pore size (0.2-0.5 µm) that affect food features (Fillaudeau & Carrère, 2002). In contrast, the supports proposed herein had a mean size that fell within the 5-50 µm range to preserve the properties of the processed beverage, and to prevent crucial factors for industrial application, such as fouling or filter cake (Gialleli, Bekatorou, Kanellaki, Nigam, & Koutinas, 2016).

Besides, the supports' size is similar or higher than the microorganisms' size allowing the pass of the cells, as can be stated by the microbial counts after filtering beer through the non-functionalized supports. Beer microbiota includes bacteria, yeast and molds. Bacterial cells are typically 0.5–5 µm in length, yeast sizes are normally 3–4 µm, whereas molds, as filamentous multi-celled fungi, present a larger size within 10–40 µm. The differences in size are in accordance with the results obtained in this study, in which the supports' removal capacity is higher for mold and yeast.

Natural antimicrobial compounds, including animal molecules like chitosan or lysozyme, bacteriocins like nisin or sakacin, and hop extracts have been applied to reduce microorganisms in beer, and have obtained remarkable antimicrobial activity against LAB, but not against yeast (Franchi, Tribst, & Cristianini, 2012; Galvagno, Gil, Iannone, & Cerrutti, 2007; Gil, del Mónaco, Cerrutti, & Galvagno, 2004). In this work, EOCs were chosen as bioactive compounds due to their reported antimicrobial properties and the fact that they

are considered GRAS molecules (Burt, 2004), although their potential in fermented beverages preservation has scarcely been explored (Chavan & Tupe, 2014). The application of these bioactive compounds presents some limitations, like their strong sensory properties and their interactions with food components (Hyldgaard, Mygind, & Meyer, 2012). In this context, the immobilization of these molecules overcomes limitations compared with their application in the free form, giving rise to novel effective antimicrobial supports (Ruiz-Rico et al., 2017).

The evaluation of the EOC-functionalized supports to remove a model microorganism from inoculated pasteurized beer showed the effectiveness of the filtration technology to reduce at least 4 log cycles of *E. coli*. This reduction level fulfils the minimum requirements for microbiologically safe non-thermal processing of beer on a commercial scale, according to brewing specialists (Walkling-Ribeiro et al., 2011). The supports with mean intermediate size (10-25 µm) were the most effective filtering materials. While filtration through the 5 µm-supports may be inefficient because of the creation of preferential paths to facilitate the flux diminishes the contact between the EOCs and the microbial cells, the use of 50 µm-particles can result in a high speed of the beer flux through the particles that decreases bacterial removal. In the same manner, this study have evidenced the ability of the different EOC-functionalized supports to remove the natural microbiota of craft beer.

Regarding the studied bioactive compounds, the terpenoids of the *Lamiaceae* family plants (thymol and carvacrol) were the most effective EOCs, resulting in total removal of beer microbiota. These results agree with previous studies that have shown the good *in vitro* antimicrobial activity of carvacrol and thymol against pathogenic and spoilage microorganisms (Abbaszadeh, Sharifzadeh, Shokri, Khosravi, & Abbaszadeh, 2014; Rota, Herrera, Martínez, Sotomayor, & Jordán, 2008). Besides, vanillin and eugenol present effective inhibitory properties against microorganisms present in different food matrices (Holley & Patel, 2005).

The pre-conditioning of filters preserved, and even improved, the removal capacity of the materials. The enhancement of the supports' retention capability after washing may be due to particle bed compaction and to the consequent increase in the filtration time that favored the contact between microorganisms and antimicrobial compounds. In addition to this, it is important to highlight that the pre-conditioning allowed us to obtain a zero wash-out effect after filtering beer with the immobilized EOCs. The preservation of the removal properties after washing and the fact that EOCs cannot be detected in beers

filtered through pre-conditioned filters, supports the premise of the covalent grafting of the bioactive compounds onto the supports' surface.

Since it has been demonstrated the absence of the EOCs in beer filtered, at the same time that the filtration process was efficient in the microbial stabilization of craft beer, it has been confirmed that the antimicrobial effect resides in the immobilized EOCs. The removal capability of the EOC-functionalized particles was probably due to the combination of two factors: the retention of the microbial cells in the bed, and to the interaction between the immobilized EOCs and the microbial cell membrane, which facilitates microbial inactivation. EOCs affect the permeability of the external microbial membrane by favoring proton flow, and altering enzymatic and energy production systems, which leads to cell death, according to other authors (Burt, 2004; Hyldgaard et al., 2012).

Processing beer with the filtration technology proposed herein allowed the microbial elimination of beer microbiota and achieved comparable results to those obtained by other non-thermal pasteurization techniques, such as pulsed electric fields (Walkling-Ribeiro et al., 2011). Processing of beer by filtration with EOC-functionalized supports, achieved adequate microbial reductions for the selected microorganisms (4-log reduction), meeting the minimum requirements for safe cold-pasteurized beer.

Whereas conventional heat pasteurization (60 °C for 15 min) affects beer properties, alternative non-thermal treatments avoid these limitations. In this study, sensory evaluation proved, mainly for the supports functionalized with thymol, the suitability of the developed processing methodology given the similarity on the attributes' scores between non-filtered and filtered beer. Therefore, this novel cold-pasteurization technology could preserve the features of craft beer and extend its shelf life better than conventional preservation methodologies.

5. Conclusions

Filtration through particles functionalized with essential oil components is an efficient methodology to reduce the microbial population present in beer. Therefore, a filtration process based on immobilized natural antimicrobial compounds has been developed as a proof of concept and has a very good potential to be used as a non-thermal preservation technique for craft beer. The developed filtering materials would be used to replace or complement the conventional filtering processes that take places in the brewing industry for clarification and stabilization of beer. However, before being applied in a real scenario, it is

necessary to study the retention capability against different spoilage and pathogenic microorganisms as well as to evaluate the influence of treatment on beverage properties.

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