UNIVERSITAT POLITÈCNICA DE VALÈNCIA
DEPARTAMENTO DE TECNOLOGÍA DE ALIMENTOS

Development of polymeric and silica filtering materials functionalized with antimicrobial compounds for the elimination of microorganisms in liquid foods

Submitted by

Nataly Peña Gomez

PhD Supervisors:

Prof. José Manuel Barat Baviera
Dr. Isabel Fernández Segovia
Dr. María Ruiz Rico

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JOSÉ MANUEL BARAT BAVIERA, PhD in Food Technology and Professor at the *Universitat Politècnica de València*, ISABEL FERNÁNDEZ SEGOVIA, PhD in Food Technology and Lecturer at the *Universitat Politècnica de València*, and MARÍA RUIZ RICO, PhD in Food Technology

CERTIFY:

That the work *"***Development of polymeric and silica filtering materials functionalized with antimicrobial compounds for the elimination of microorganisms in liquid foods"** has been developed by Nataly Peña Gomez under their supervision in the Departamento de Tecnología de Alimentos of the *Universitat Politècnica de València*, as a Thesis Project in order to obtain the degree of PhD in Food Technology at the *Universitat Politècnica de València.*

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Prof. José Manuel Barat Baviera Prof. Isabel Fernández Segovia Dr. María Ruiz Rico

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"Llegar juntos es el principio.
Mantenerse juntos, es el progreso.
Trabajar juntos es el éxito."
Henry Ford

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ABSTRACT

In the present doctoral thesis the use of new cellulosic and silica supports as filtering systems for the stabilization and preservation of liquid foods has been evaluated to overcome two major challenges of the beverage industry. On the one hand, avoid or minimize the changes in the nutritional, structural and organoleptic properties of food caused by traditional thermal pasteurization, and offer an alternative to the problem of low viability due to high investment/production costs when applying new non-thermal technologies. Therefore, this doctoral thesis focuses on the development and evaluation of a new non-thermal technology for the preservation of liquid foods based on filtration. The filtering systems have been developed from cellulosic and silica supports, non-modified or functionalized with antimicrobial compounds.

In the first chapter, the use of cellulose materials as filtering supports for the treatment of liquid foods was evaluated. As first approximation, a porous nano-micro tubular material was developed from the extraction and delignification of the cellulosic material present in the corn stalk. The use of this support was effective as filtering material for the treatment of water and orange juice, in a continuous flow system, eliminating the microbial load. The application of this support as filtering system has several advantages, such as its microbial retention capacity, the reuse of corn by-products and, therefore, its respect for the environment. However, it would be necessary to optimize the filtering process to avoid the frequent clogging of its pores that required several washing cycles during the process, as well as to establish a method of material regeneration to increase its life. In addition, this system affected the color of the filtered juice, which did not remain constant during the process, representing an important disadvantage that must be addressed. As a second approach, the potential of the immobilization of a bioactive molecule on cellulose membranes was evaluated to improve the microbial retention capacity of the cellulosic material, as well as to allow its reuse. The cellulose filters functionalized with polyamines proved to be effective in eliminating pathogens in water, due to the positive charges generated by the amine groups immobilized on

the surface of the membranes, which attract and retain the negatively charged bacteria. Given the easy preparation and usage of the polyamines-functionalized cellulose membranes, these could be considered a good option for the development of fast, easy to use and low cost *in situ* water treatment systems.

The second chapter describes the development and application of silica particles functionalized with essential oil components to design filtering aids with antimicrobial activity. The filtration of various food matrices (water, beer and apple juice) through the supports functionalized with natural antimicrobials proved to be effective in reducing the load of the pathogenic strain *Escherichia coli*, as well as reducing the endogenous microflora of beer and the juice (lactic acid bacteria, mesophilic, psychrophilic, mold and yeast). The removal capability is due to the combination of physical adsorption and contact inactivation with the essential oil compounds immobilized. In addition, the evaluation of the physicochemical and sensory properties of the liquid foods studied showed a not significant effect, it depends on the size of the silica particles used and the immobilized bioactive molecule. Therefore, the proposed preservation system has a high potential for cold beverage pasteurization processes.

Resumen

En la presente tesis doctoral se ha evaluado el uso de nuevos soportes celulósicos y silíceos como sistemas de filtración para la estabilización y conservación de alimentos líquidos con el fin de afrontar dos grandes retos de la industria de bebidas. Por un lado, evitar o minimizar los cambios en las propiedades nutricionales, estructurales y organolépticas de los alimentos, ocasionados por la pasteurización térmica tradicional, y ofrecer una alternativa al problema de la baja viabilidad debida a los altos costos de inversión/producción al aplicar nuevas tecnologías no térmicas. Por ello, esta tesis doctoral se centra en el desarrollo y evaluación de una nueva tecnología no térmica de conservación de alimentos líquidos basada en la filtración. Se han desarrollado sistemas de filtración a partir de soportes celulósicos y silíceos, sin funcionalizar o funcionalizados con compuestos antimicrobianos.

En el primer capítulo se evaluó el uso de materiales de celulosa como soportes filtrantes para el tratamiento de alimentos líquidos. Como primera aproximación se desarrolló un material poroso nano-micro tubular a partir de la extracción y deslignificación del material celulósico presente en el corazón o raquis de la mazorca de maíz. El uso de este soporte resultó ser efectivo como material filtrante para el tratamiento de agua y zumo de naranja, en un sistema de flujo continuo, eliminando la carga microbiana. La aplicación de este soporte como sistema de filtración presenta diversas ventajas como su capacidad de retención microbiana, la reutilización de sub-productos del maíz y, por tanto, su respeto al medioambiente. Sin embargo, sería necesario optimizar el proceso de filtrado para evitar la frecuente obturación de sus poros que requirió varios ciclos de lavado durante el proceso, así como establecer un método de regeneración del material para incrementar su vida útil. Además, este sistema afectó al color del zumo filtrado, que no se mantuvo constante durante el proceso, lo que supone una importante desventaja que es necesaria abordar. Como segunda aproximación, se evaluó el potencial de la inmovilización de una molécula bioactiva sobre membranas de celulosa, para mejorar la capacidad de retención microbiana del

material celulósico, así como permitir su reutilización. Los filtros de celulosa funcionalizados con poliaminas demostraron ser eficaces en la eliminación de patógenos en agua, debido a las cargas positivas generadas por los grupos amina inmovilizados en la superficie de las membranas, que atraen y retienen las bacterias cargadas negativamente. Dada la fácil preparación y procedimiento de uso de las membranas de celulosa funcionalizadas con poliaminas, éstas podrían ser consideradas una buena opción para el desarrollo de sistemas de tratamiento de aguas *in situ*, rápidos, de fácil manejo y de bajo coste.

El segundo capítulo describe el desarrollo y aplicación de partículas de sílice funcionalizadas con compuestos de aceites esenciales, con el fin de diseñar coadyuvantes de filtración con actividad antimicrobiana. La filtración de diversas matrices alimentarias (agua, cerveza y zumo de manzana) a través de los soportes funcionalizados con los antimicrobianos naturales demostró ser eficaz en la reducción del recuento de la cepa patógena *Escherichia coli*, así como frente a la microflora endógena de la cerveza y el zumo (bacterias acidolácticas, aerobios mesófilos, psicrófilos, mohos y levaduras). La eficacia en el control microbiano se debe a la combinación de la adsorción física y la inactivación por contacto con los compuestos de aceites esenciales inmovilizados. Además, la evaluación de las propiedades físico-químicas y sensoriales de los alimentos líquidos demostró un efecto poco significativo, éste depende del tamaño de las partículas de sílice usadas y de la molécula bioactiva inmovilizada. Por lo tanto, el sistema de conservación propuesto tiene un alto potencial para procesos de pasteurización en frío de bebidas.

RESUM

En la present tesi doctoral s'ha avaluat l'ús de nous suports cel·lulòsics i silicis com a sistemes de filtració per a l'estabilització i conservació d'aliments líquids, amb la finalitat d'afrontar dos grans reptes de la indústria de begudes. D'una banda, evitar o minimitzar els canvis en les propietats nutricionals, estructurals i organolèptiques dels aliments, ocasionats per la pasteurització tèrmica tradicional, i oferir una alternativa al problema de la baixa viabilitat deguda als alts costos d'inversió/producció en aplicar noves tecnologies no tèrmiques. Per això, aquesta tesi doctoral es centra en el desenvolupament i avaluació d'una nova tecnologia no tèrmica de conservació d'aliments líquids basada en la filtració. S'han desenvolupat sistemes de filtració a partir de suports cel·lulòsics i silicis, sense funcionalitzar o funcionalitzats amb compostos antimicrobians.

En el primer capítol es va avaluar l'ús de materials de cel·lulosa com a suports filtrants per al tractament d'aliments líquids. Com a primera aproximació es va desenvolupar un material porós nano-micro tubular a partir de l'extracció i deslignificació del material cel·lulòsic present en el cor o raquis de la panolla de dacsa. L'ús d'aquest suport va resultar ser efectiu com a material filtrant per al tractament d'aigua i suc de taronja, en un sistema de flux continu, eliminant la càrrega microbiana. L'aplicació d'aquest suport com a sistema de filtració presenta diversos avantatges com la seua capacitat de retenció microbiana, la reutilització de subproductes de la dacsa i, per tant, el seu respecte al medi ambient. No obstant això, seria necessari optimitzar el procés de filtrat per a evitar la freqüent obturació dels seus porus que va requerir diversos cicles de rentada durant el procés, així com establir un mètode de regeneració del material per a incrementar la seua vida útil. A més, aquest sistema va afectar el color del suc filtrat, que no es va mantenir constant durant el procés, la qual cosa suposa un important desavantatge que és necessari abordar. Com a segona aproximació, es va avaluar el potencial de la immobilització d'una molècula bioactiva sobre membranes de cel·lulosa, per a millorar la capacitat de retenció microbiana del material cel·lulòsic, així com permetre la seua reutilització. Els filtres de cel·lulosa funcionalitzats amb

poliamines van demostrar ser eficaces en l'eliminació de patògens en aigua, a causa de les càrregues positives generades pels grups amina immobilitzats en la superfície de les membranes, que atrauen i retenen els bacteris carregats negativament. Donada la fàcil preparació i procediment d'ús de les membranes de cel·lulosa funcionalitzades amb poliamines, aquestes podrien ser considerades una bona opció per al desenvolupament de sistemes de tractament d'aigües *in situ*, ràpids, de fàcil maneig i de baix cost.

El segon capítol descriu el desenvolupament i aplicació de partícules de sílice funcionalitzades amb compostos d'olis essencials, amb la finalitat de dissenyar coadjuvants de filtració amb activitat antimicrobiana. La filtració de diverses matrius alimentàries (aigua, cervesa i suc de poma) a través dels suports funcionalitzats amb els antimicrobians naturals va demostrar ser eficaç en la reducció del recompte del cep patogen *Escherichia coli*, així com enfront de la microflora endògena de la cervesa i el suc (bacteris àcid làctics, aerobis mesòfils, psicròfils, floridures i llevats). L'eficàcia en el control microbià es deu a la combinació de l'adsorció física i la inactivació per contacte amb els compostos d'olis essencials immobilitzats. A més, l'avaluació de les propietats fisicoquímiques i sensorials dels aliments líquids estudiats va demostrar un efecte poc significatiu, aquest depèn de la grandària de les partícules de sílice usades i de la molècula bioactiva immobilitzada. Per tant, el sistema de conservació proposat té un alt potencial per a processos de pasteurització en fred de begudes.

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1. PREAMBLE

1. PREAMBLE

This PhD thesis forms part of the projects "***Hybrid systems based on biocompatible supports for development of antimicrobials based on naturals substances and controlled release*** (AGL2015-70235-C2-1-R)", funded by the 2015-2018 National Research Plan of the Spanish Ministry of Economy and Competitiveness; and **"*Development and application of antimicrobial systems for the food industry based on functionalized surfaces and controlled release systems*"** (RTI2018-101599-B-C21), funded by the Ministerio de Ciencia, Innovación y Universidades, the Agencia Estatal de Investigación and FEDER-EU.

The main objective of the first project was to develop particles with antimicrobial activity by anchoring naturally-occurring antimicrobials, and to develop new and cheaper smart delivery systems based on hybrid materials for controlled release in the digestive tract. The main objective of the second project was to develop new antimicrobial systems based on the encapsulation or covalent immobilization of naturally-occurring antimicrobial compounds on several materials for their application in different steps of the food processing industry.

The growing consumers' demand for high quality food, free of synthetic preservatives has forced in recent years the development and application of new preservation methodologies and antimicrobial agents. In this context, the design of functionalized particles based on the covalent grafting of essential oil components has been carried out in these projects, resulting in antimicrobial particles that can be used as food preservatives. These preservatives based on the functionalized particles presented the antimicrobial properties of the immobilized natural antimicrobials with minimal effect on the food sensory properties, unlike the free essential oil components. Besides the use of the antimicrobial particles as food preservatives, the functionalized supports may be used as filtering aids in the beverage industry to microbiologically stabilize liquid food as an alternative to conventional preservation methodologies.

Previous doctoral theses based on the development and application of novel hybrid systems with antimicrobial properties have been undertaken in the previous years to cover the mentioned objectives of the projects. Thus, the doctoral thesis entitled “Development of polymeric and silica filtering materials functionalized with antimicrobial compounds for the elimination of microorganisms in liquid foods” is the fourth doctoral thesis related with these projects.

2. GENERAL INTRODUCTION

1. Conventional disinfection and preservation methods

Food safety is essential to prevent food-borne illnesses. Ensuring the hygienic quality is mandatory for food operators and, for this reason, preservation treatments are often needed in the food industry.

The main pollutants in water are classified as physical, chemical and biological contaminants. Sediments, radioactive substances and high temperature are the main physical polluting agents. Among the chemical contaminants, pesticide and fertilizer residues are some of the most important. Lastly, vegetable contaminants, parasites and microorganisms are considered biological contaminants (Arcos-Pulido et al., 2017). Chlorine disinfection has been recognized as one of the greatest achievements in the field of public health of the 20th century (CDC, 1999). However, the presence of by-products derived with a possible carcinogenic connection has been the cause of the modifications of the quality standards of drinking water, specifically, with regard to the maximum acceptable concentration of trihalomethanes (THMs) (Camenforte & Pérez, 2014). The use of alternative disinfectants to chlorine, such as chloramine, chlorine dioxide or ozone, also generates harmful disinfection by-products (Rodríguez et al., 2007). The use of chloramine is associated not only with the formation of THMs and haloacetic acids, but also with the formation of nitrites, nitrates, haloacetones and N-Nitrosodimethylamine. Likewise, chlorine dioxide generates chlorites and chlorates; while the use of ozone generates bromates, aldehydes, biodegradable organic carbon, ketoaldehyde acids, bromoforms, peroxides and epoxides (Vinette, 2001).

The stability of more complex food liquid matrices like milk, juice or low-alcoholic drinks depends largely on the growth of microorganisms, or the oxidation of bioactive compounds. Different preservation techniques can be used to reduce or eliminate these causative agents, including methods such as pasteurization, drying, freezing, irradiation, and the addition of chemical additives, among others. Thermal pasteurization and sterilization are the most common techniques currently used to inactivate microorganisms in food products (Piyasena et al., 2003). The

success of the heating methodologies is due to their efficiency to inactivate the microbial load, environmentally friendly use and preservative-free technique. However, these processes produce nutritional loss, cause undesirable sensorial changes and deteriorate some functional properties of food products (Chemat et al., 2011; Morris et al., 2007).

The limitations of conventional methods of disinfection and preservation commonly used to stabilize water and beverages, promote the search for alternative methods to ensure the microbial safety of liquid foods, maintaining the food quality in an economical way.

2. Non-thermal preservation methods

Consumers demand for high quality fresh foods, free form chemical preservatives, with an extended shelf-life, produced with sustainable methods and small carbon footprints (Koutchma, 2009). Therefore, alternative processing methodologies that do not implies heat treatment are being investigated to assure microbiologically stable foods and to preserve the sensory and nutritional quality of the fresh-like food products (Gialleli et al., 2016).

In recent years, the definition of pasteurization for food has been revised, including any process, treatment, or combination thereof, which is applied to food to reduce the majority of microorganisms of public health significance (Koutchma, 2009). This definition comprises different non-thermal pasteurization processes, such as high pressure processing (HPP), pulsed electric fields (PEF), irradiation and pulsed light treatment (PL), ultrasound (US), dense phase carbon dioxide (DPCD), ozone and filtration (Milani & Silva, 2017; Pereira & Vicente, 2010). In addition, naturally-occurring antimicrobial compounds such as essential oils, animal enzymes and fatty acids or bacteriocins, among others, can be used to provide microbial inactivation in food products (Corbo et al., 2009).

2.1. High pressure processing

High pressure processing (HPP) is a non-thermal technology applied in food industry since 1990 (Roobab et al., 2018), based on the application of high pressure (100 to 1000 MPa) to food products in order to inactivate vegetative microbial cells (Daryaei et al., 2016). With HPP different solid or liquid food products can be preserved. The most liquid food subjected to this treatment are fruit and vegetable juices, and alcoholic beverages (Mújica-Paz et al., 2011).

HPP treatment affects non-covalent bonds, such as van der Waals forces, electrostatic interactions and hydrogen bonding, which can inactivate microorganisms by interrupting cellular functions and producing protein conformation changes and membrane perturbation (Mok et al., 2006; Mújica-Paz et al., 2011). These effects eventually cause partial or complete denaturation of cell components (Roobab et al., 2018). In contrast, food treated with HPP present minimal chemical changes with maintenance of the sensory and nutritional properties of fresh like food products (Mújica-Paz et al., 2011). Besides, HPP can improve some organoleptic properties of beverages such as beer or wine (Buzrul, 2012). HPP causes a fall in the pH and then foodstuffs with acid pH are good candidates for this non-thermal treatment (Pereira & Vicente, 2010). However, HPP treatment affects the secondary and tertiary structures of macro-molecules, such as proteins and polysaccharides, which can modify texture in foods and may produce loss in nutritional and functional food properties (Mújica-Paz et al., 2011; Roobab et al., 2018).

Although this technology is able to inactivate the vegetative forms of pathogenic and spoilage bacteria, the inactivation of bacterial spores by pressure alone is not possible (Mújica-Paz et al., 2011). Some studies have obtained good results on the inactivation of microorganisms by combination of HPP with thermal processes (Morris et al., 2007).

Despite the great opportunities offered by HHP for food preservation, this technology has some limitations, such as the presence of vegetative bacteria

resistant to pressure, the modification of rheological properties of the food products and the high investment costs (Garcia-Gonzalez et al., 2007).

2.2. Pulsed electric fields

Pulsed electric fields (PEF) technology is based on the application of short bursts high voltage (10–80 kV/cm) into foods placed between two electrodes, for less than one second (Pereira & Vicente, 2010). This process can be applied to pumpable fluid or semi-fluid foods such as juices, milk, liquid eggs, soups or brines. PEF is an efficient process that is employed to inactivate microorganisms and decrease the activity of enzymes without major undesirable effects on the organoleptic qualities of food products (Lasekan et al., 2017). PEF causes damage on the microbial cell membranes (electroporation) which affects essential cell functions and may lead to cell death of planktonic cells (Barba et al., 2015; Pereira & Vicente, 2010).

PEF origins minimal negative changes in sensory and nutritional properties in foods, with minimum loss of aroma active compounds and physical properties (Evrendilek, 2016). Besides, some studies have demonstrated the ability of PEF methodology to increase the extraction of some bioactive compounds like phenolic compounds or vitamins in beverages, improving their bioaccessibility (Barba et al., 2015).

Enzymes are not significantly affected by PEF processing and studies show controversial results, because the mechanism of changes in protein structure is not yet clear (Soliva-Fortuny et al., 2009). PEF technology cannot inactive enzymes and treated food should be distributed refrigerated (Van-Loey et al., 2001). A combination of PEF and mild heat may inactivate enzymatic activity during food storage (Pereira & Vicente, 2010). In addition, the combination of PEF with thermal treatment may inactivate bacterial endospores (Barba et al., 2015).

Despite the advantages of this technology, the main limitation of PEF is the potential release of metals form electrical materials, due to corrosion and migration, which can affect the flavour of the treated food products (Yang et al.,

2016). Besides, PEF cannot be used on food products that contain or could form air bubbles, which limited its application in the food industry (Morris et al., 2007).

2.3. Irradiation

Irradiation is an established disinfection method used to eliminate spoilage and pathogenic microorganisms from air, surfaces, drinking water and liquid food products, such as fruit juices (Choi & Nielsen, 2005; Keyser et al., 2008; Pereira & Vicente, 2010). Irradiation damages DNA which destroys essential functions of the microbial cells, such as reproduction (Tiwari et al., 2009).

Irradiation methods can be from different electromagnetic spectrum, such as radioisotopic ($Cobalt^{60}$ or $Cesium^{137}$), electrons, X-rays, or ultraviolet (UV) sources (Morris et al., 2007).

The efficacy of this method depends on the penetration capability of irradiation through the surface of the liquid food. The penetration effect of irradiation depends on the type of liquid, its optical and physical properties, the chemical composition, and the amount of soluble solids in the liquid (Koutchma, 2009). Therefore, microorganisms suspended in water are more sensitive to irradiation than microorganisms in juices (Keyser et al., 2008).

Irradiation does not induce significant losses of nutrients and sensory quality in food because the temperature of the food is maintained during processing (Song et al., 2007; Tiwari et al., 2009). Non-toxic by-products are formed during the irradiation treatment when compared to other pasteurization methods (Keyser et al., 2008).

However, it has been reported that some protein food products treated with this methodology can develop off-flavour, odour and colour (Odueke et al., 2016). Besides, some nutrients, such as vitamins, antioxidants, unsaturated fatty acid and phospholipids are light-sensitive compounds, and the irradiation treatment can produce loss of nutrients in processed beverages (Koutchma, 2009). Other

disadvantage to take into account is the potential health problem for industry workers exposed to irradiation source (Pereira & Vicente, 2010).

2.4. Pulsed light

Pulsed light (PL) technology consists of short time high-peak pulses of broad spectrum white light to eliminate microbial contamination of surfaces and food products. This method has emerged as an alternative to continuous ultraviolet irradiation treatment to improve penetration depth and emission power(Kasahara et al., 2015). However, PL treatment is more effective for the disinfection of surfaces than liquid food products, because of the low UV transmittance of liquid foods (Ansari et al., 2019). Other current limitations of this technology are the high investment cost and the technological problems to avoid food overheating (Oms-Oliu, Martín-Belloso, & Soliva-Fortuny, 2010).

2.5. Ultrasound

Ultrasound (US) can be used as a food preservation method to destroy bacteria, molds and yeasts, and to inactivate enzymes by cell lysis (Milani & Silva, 2017). Ultrasound in a liquid induces bubble cavitation which result in localized high temperature and pressure that disintegrate biological cells and denature enzymes. The mechanism of microbial killing is mainly due to the thinning of cell membranes, localized heating and production of free radicals (Chemat et al., 2011; Tiwari et al., 2009).

The effectiveness of the ultrasound depends on the acoustic frequency, temperature and pressure applied. In combination with heat and/or pressure, this technology can improve the sterilization efficiency, but it can be used alone for some liquid food, such as fruit juices, sauces, purees and dairy products (Chemat et al., 2017). US has been proved to be effective for the elimination of pathogens microorganisms with no significant changes in sensory properties and nutrients

content of treated food. US also inactivates enzymes responsible for the deterioration of beverages quality and can improve the dispersion and microbial stability in fruit-vegetable juices (Ferrario & Guerrero, 2016; Ó. Rodríguez et al., 2018) and beer (Milani & Silva, 2017).

One of the major disadvantages of US is that the presence of small gas bubbles in a sample can disturb the propagation of US through the sample and reduce the efficacy. Another potential problem is that a lot of information about the thermophysical parameters of a material is needed in order to make theoretical predictions of its ultrasonic properties (Mantelet et al., 2019).

2.6. Dense phase carbon dioxide

Dense phase carbon dioxide (DPCD) technique consists of submitting food products to pressurized CO_2 (10–20 MPa) affecting the viability of microorganisms and the functionality of enzymes (Amaral et al., 2017). DPCD has been shown to eliminate vegetative forms of spoilage and pathogenic microorganisms. This technology inactivates microorganisms by several mechanisms such as oxygen elimination, lowering of intracellular and extracellular pH, enzyme inhibition or physical disruption and modification of cell membranes (Jan et al. , 2017). DPCD can also inactivate certain enzymes, which cause quality deterioration in fruit and vegetable juice processing, due to pH lowering, conformational changes, and inhibitory effect of molecular CO_2 on enzyme activity (Damar & Balaban, 2006; Villamiel et al., 2017).

The DPCD treatment has been mainly studied in juices, obtaining beverages without significant changes on their nutritional and sensory properties (Bevilacqua et al., 2018).

2.7. Ozone

Ozone treatment within the food industry has been performed on solid foods by either gaseous processing or washing with ozonated water, but after the FDA approval of ozone as a direct additive to food, this compound can be used to the disinfection of liquid foods by ozonation (Pandiselvam et al., 2019). Ozone is considered an antimicrobial agent against bacteria, fungi, viruses and spores(Reddy et al., 2017). The inhibitory activity of ozone is due to its strong oxidizing activity and its capability to diffuse through cell membranes which cause damage of cellular membranes and essential components such as proteins and DNA (Ghosh et al., 2018).

Some studies have shown that ozonation of fruit juices rich in anthocyanins causes a significant reduction in these antioxidant compounds due to the strong oxidizing activity of the ozone(Jiménez-Sánchez et al., 2017). In addition, ozone produces disinfection by-products (DBPs) such as formaldehyde and acetaldehyde, which are recognized as mutagenic substances (Ikehata, 2019)

2.8. Filtration

Filtration involves an important non-thermal process in food industry for clarification, concentration and microbial stabilization of beverages (Gialleli et al., 2016).

Membrane filtration based on materials such as polysulfones, polypropylene, polyamide, nylon or cellulose acetate are used in food industry for complete or partial removal of microbes in beer, wine and juices, but membrane fouling and cleaning methods are critical factors for the extensive application of this technology (Gialleli et al., 2016). The ceramic membranes from naturally occurring minerals (zeolite, apatite, clays, kaoline, dolomite, etc.) are used for beverages clarification with low cost (Rascon-Escajeda et al., 2018). On the other hand, bed filtration based on sand or diatomaceous earth is used for the removal of organic matter and microorganisms from water and liquid foods, but these do not fulfill the needed

efficiency in removing pathogens and present regeneration/disposal issues (Devi et al., 2008).

Filtration through a porous membrane results in the accumulation of solid particles and/or cells from the liquid food on the membrane as a cake, which decreases the flow rate of the fluid due to the filter resistance. The filter resistance produced by cells cake depends on the morphology of the microbial cells being higher for bacteria than bigger microorganisms such as yeasts (Lemma et al., 2015).

Filtration can be generally classified according to the pore size of the membrane (Figure 1). The main filtration techniques used to remove organic matter, microorganisms and contaminants from water and liquid food are described in the following subsections, highlighting the procedure principle, efficiency, applicability and cost.

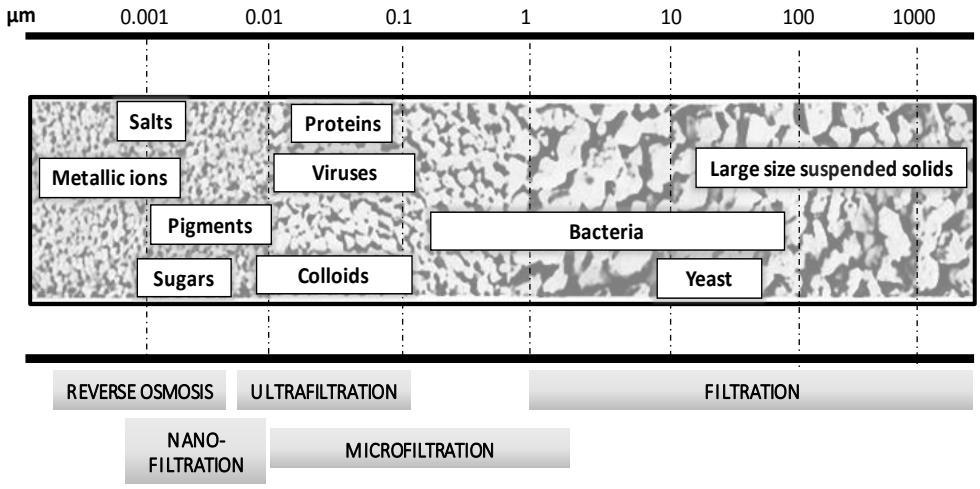

Figure 1. Types of filtration according to pore size. Adapted from Palacio Martinez (1999) and Jimenez Feliz, Lora Garcia and López Pérez (2017)

2.8.1. Standard filtration

2.8.1.1. Slow sand filtration

Sand has been used for around 150 years as a relatively simple and easy-to-operate process that allows raw water to pass through a sand medium. As the water passes through the sand, solids, microorganisms and heavy metals are removed. Slow sand filtration (SSF) can be simply and easily operated for pathogen removal from water, such as *Escherichia coli*, *Enterococci*, *C. perfringens* spores, coliphages and heterotrophic bacteria (Seeger et al., 2016). Water percolates through a sand column, on the surface of which a biologically active compartment is formed where most of the pathogen removal takes place. Pathogen retention is produced by adsorption, while pathogen inactivation is caused by abiotic (starvation) and biotic mechanisms (predation by eukaryotic bacterivores and lysis induced by bacteriophages and algal-derived reactive oxygen species) (Pfannes et al., 2015).

However, extended large areas per unit volume of water treated are needed. Besides, excessive suspended solids can clog the filters and the presence of contaminant such as pharmaceutically active compounds or high salinity can negatively impact on the ability to remove bacteria (Hoslett et al., 2018).

2.8.1.2. Rapid sand filtration

Unlike SSF, rapid sand filtration (RSF) produces no significant biological layer. Hence, this can be alternatively applied along with a certain prior and post treatment to remove pathogenic substances and prevent fouling. Coating the sand filters with metal-based additives (e.g. Fe or Mn) is a relatively easy process that can significantly increase their adsorption potential. Nevertheless, RSF shows mixed results with regards to the WHO guidelines. In few cases, the remaining contaminant concentration meets the requirements (Hoslett et al., 2018).

2.8.1.3. Activated carbon

Granular activated carbon (GAC) is mainly used in water disinfection. GAC has a high adsorption capacity of organic matter and heavy metal ions. Due to these properties, GAC can increase the chemical demand of oxygen and the efficiency in the electrical conductivity removal in water (Kose-Mutlu et al., 2017). GAC also present high adsorption capacities of cyanotoxins and can support high amounts of heterotrophic and nitrifying biomass, obtaining a better final taste, odor and color in water. In spite of the high cost, GAC can be produced from waste-dust, and their combination with other methods (e.g. microfiltration and slow sand filtration) can constitute a cost-effective way to produce acceptable water drinking quality. However, the lifecycle of a GAC filter is limited, because it is usually saturated with target pollutants. Biological activity in GAC generates problems such as clogging, anaerobic/dead zones and detachment of microbes from the GAC (Hoslett et al., 2018).

In beverage industry, active carbon has been used to clarify juices. However, it has the disadvantage that adsorbs some aroma, color pigments and turbid particles, resulting in a high impact on the organoleptic properties of the juices (Horvath-Kerkai & Stéger-Máté, 2012).

2.8.1.4. Cellulosic supports

Membrane technology often use polymeric membranes made of polyamide, polysulfone and polyimide. However, these membranes suffer environmental drawbacks (made from petroleum based, non-biodegradable and required large quantities of solvents and chemicals to be produced) (Klemm et al., 2011). In response to the mentioned challenges, the use of cellulose is being the best alternative, because it is the most abundant natural polymer that can be used in the form of fibers and multiple derivatives.

Depth cellulose filters are porous materials capable of removing contaminants and retain molecules not only on the surface but also within the media. In the food

industry, nanocellulose (NC) has widely been explored as an alternative polymer in membranes/filters, especially in beverages clarification, due to NC unique properties, such as high surface area, high strength, wettability, ease of chemical functionalization and low environmental impact (Onur et al., 2018). NC also has a high area-volume ratio, which increases the material absorption capacity (Salas et al., 2014).

In the last years, porous cellulosic particles or tubular cellulose material (TCM) from simple agricultural by-products delignification (e.g. sawdust, rice husk), has been developed as an alternative to conventional cellulosic membranes. TCM started to be used as biocatalyst support in wine (Agouridis et al. 2008; Agouridis et al., 2005) and to accelerate olive ripening (Papafotopoulou-Patrinou et al., 2015). But more important, the use of TCM as filtering material was evaluated in microbial retention capacity studies, to develop a renewable continuous preservation system for water (Kumar et al., 2016), apple juice (Gialleli et al., 2016) and wine (Gialleli et al., 2014). This proposed non-thermal pasteurization technology was effective removing *Saccharomyces cerevisiae, Acetobacter pasteurianus, Lactobacillus bulgaricus* and *Lactobacillus plantarum*. The cellular immobilization in TCM is due to the physical adsorption by hydrogen bonding, other weak forces, as well as to the natural capture in the pores of the material (Koutinas et al., 2012). Despite the promising results, TCM presents some limitations for its use as filtering element. This material requires several regeneration cycles due to the frequent obturation of the supports. In addition, the high filter length and TCM compression limits the flow of viscous liquid foods. Finally, the natural degradation of the cellulosic matrix implies a short life cycle.

2.8.1.5. Bentonite

Bentonite is a clay composed by montmorillonite and/or other terms of the smectite group, and minor non-clay minerals. Montmorillonite has a high cation exchange capacity, marked swelling and high specific surface area leading to a strong adsorption/desorption capacity (de Mattos et al., 2017). Bentonite presents several advantages, such as low cost, environmental friendliness, stability and reusability (Boz et al., 2013; Sasikala et al., 2014).

Ceramic membranes make with bentonite have been used for clarification of wine (Armada & Falqué, 2007) and fruit juices (Urošević et al., 2017), specially to remove the dark color of apple juice (Oszmiański & Wojdyło, 2007; Youn et al., 2004).

In the food industry, the use of natural bentonite presents opposing views. Bentonite as filtering material is effective to obtain protein stability in beverages. It also improves fermentation rate and clarification of juices. However, the filtration through bentonite can induce loss of several volatile compounds, phenolic substances and color, especially in wine (Mierczynska-Vasilev & Smith, 2015).

2.8.1.6. Diatomaceous earth

Diatomaceous earth (DE) are unicellular photosynthetic algae enclosed in porous 3D nanopatterned silica enclosures called “frustules”, which exhibit similar properties as synthetic materials, generally used for filtering applications. After mining, DE is crushed, milled, dried, calcined and classified into different sizes in the range 1.5–22.0 μm. DE is featured by unique hierarchical porous structure with high surface area, tailorable surface modification together with high biocompatibility and chemical stability (Maher et al., 2018).

DE has been used for water pre-treatment because it is simple to operate and effective removing algae, pesticides and asbestos from water (Bhardwaj & Mirliss, 2005). Besides, diatomite is the most widely used filter aid in wine and spirit

filtration because of its low price and good filtering behavior. However, DE is not an inert filter aid and its use can involve the release of amorphous silica and silicon, calcium sulphate and some heavy metals (Gómez et al., 2014).

DE filtration is known to be the standard operation in brewing industry for the final filtration of beer (Esmaeili et al., 2015). However, this process was challenged in the last several years because of some serious environmental, sanitary and economic considerations (Conidi et al., 2015). From the environmental point of view, the diatomaceous earth is recovered from open-pit mines and constitutes a natural and finite resource. After being used in the filtration, recovery, recycling and disposal are a major difficulty due to their polluting effect. From the health perspective, the used diatomaceous earth is classified as 'hazardous waste' before and after filtration (WHO defines the crystalline silica as a cause of lung disease) and its use requires ensuring safe working conditions. From an economic standpoint, the diatomaceous earth consumption and sludge disposal generate the main cost of the filtration process (Fillaudeau et al., 2006).

In order to reduce the mentioned impacts and approach DE biocompatibility and chemical stability, considerable research efforts have been devoted to modify diatom silica structure into technologically more suitable functional materials by incorporation of inorganic oxides (MgO, TiO_2, zeolites), metals (Au) and polymers (polyaniline) (Yu et al., 2012). Some modifications of DE have resulted in the improvement of their retention capacity, such as the decrease of the negative charge of DE, increasing its ability to adsorb viruses in water (Farrah et al., 1991; Michen et al., 2012).

2.8.1.7. Silica particles

Silica particles (SP) have a diverse range of industrial and scientific applications including adsorption, catalysis, humidity control and drug delivery, among others (Zůza et al., 2019). They possess high stability and durability, and are easily

modifiable by the established organosilane chemistry, allowing the incorporation of an array of functional groups (Agnihotri et al., 2015).

In the food sector, they can be found as food additives (E-551) like anticaking agents in salt, spices or instant soups, as flavor enhancers or food pigments, as coating material in confectionary products and packaging materials or as health supplements (Wittig et al., 2017).

The silica gel has been used as filtering material, especially to clarify beverages. The silica has the capacity of binding to the polyphenols and proteins that cause turbidity. Thus resulting in a colloidal structure that precipitates, clarifying the beverage. But the efficiency of filtration depends on the food matrix. For example, it is more effective to remove proteins in beer than in apple juice, due to the polyphenol-rich environment of apple juice, which affects the chemical interactions, since there is competitiveness between proteins and polyphenols for binding to the silica surface (Siebert & Lynn, 1997). In order to equilibrate the chemical-competitive effect mentioned above, silica gel is more effective used in combination with polyvinylpolypyrrolidone (PVPP) to clarify beer(Peterson, 2003).

On the other hand, silica sol has been used for clarification in the fruit juice industry. Silica sol is the aqueous colloid solution of polymerized silica anhydride, which contains negatively charged particles that absorb positively charged proteins (Sinha et al., 2012). It has been shown that clarification efficiency of silica sol is significantly improved by combination with gelatin. This mixture was used in cherry juice processing, obtaining better results than using only silica sol, because the gelatin is a protein-based clarifying agent that precipitates negatively charged particles (Meyer et al., 2001).

2.8.2. Microfiltration and ultrafiltration

Microfiltration (MF) and ultrafiltration (UF) involve the separation of larger macromolecules in which mainly a sieving process takes place as mechanism of transport. In food industry, the main purpose of MF is to clarify beverages by

removal of suspended solids, fat and high molecular weight proteins (Vu, Darvishmanesh, Marroquin, Husson, & Wickramasinghe, 2016). The UF capacity to retain proteins has been used in the milk industry to recover nutrients and produce improved cheese (Ding et al., 2016); to extract and concentrate bioactive proteins, such as casein and whey proteins (Crowley et al., 2014) and to develop new infant milk formulations with increased heat stability of proteins (Crowley et al., 2015).

Besides, MF and UF can effectively remove bacteria and/or act as pretreatment before nanofiltration (NF) or reverse osmosis (RO), thus reducing the possibility of fouling in these next steps. MF and UF processes allow the preservation of beverages and dairy products preventing microbial growth. With aseptic processing and packaging, a cold-sterilized beverage could potentially be produced, such as fruit juices, beer, wine and water. However, an important limitation of MF and UF processes is the retention of bioactive compounds, such as polyphenols, sugars, vitamins and proteins responsible for the organoleptic properties of drinks (Rascon-Escajeda et al., 2018). Other relevant drawback of these techniques is the membrane fouling, negatively affecting membrane performance, filtration capacity and membrane life (Vu et al., 2016).

MF/UF membranes materials including ceramics, metals and polymers can suffer modification (e.g. with TiO_2 nanoparticles) to improve their performance.

2.8.3. Nanofiltration and reverse osmosis

NF is used for separation, concentration and purification of biofunctional molecules (Nath et al. 2018), dealcoholizing of beer and wine (Mangindaan et al., 2018) and have been extensively applied for concentration and separation of whey protein load (Kotsanopoulos & Arvanitoyannis, 2015).

In RO, a hydraulic pressure greater than the osmotic pressure must be applied for water to move from high solute to low solute concentration (Rastogi, 2018). RO is used to concentrate, purify and recover valuable components (Bhattacharjee et

al., 2017). RO process has been specially employed to clarify and preconcentrate fruit juices (Rastogi, 2018).

NF and RO membranes have great fouling problems by the small porous size (Hoslett et al., 2018). Moreover, the membranes have a limited life, which significant increases costs, and this technology requires high pressure and, consequently, lower flows are obtained, compared with other filtration processes (Kotsanopoulos & Arvanitoyannis, 2015).

2.9. Natural antimicrobials

Antimicrobials are chemical compounds that can either inhibit the growth or inactivate microorganisms (Weiss et al., 2009). Depending on their origin, antimicrobials used in the food industry can be classified into artificial or synthetic preservatives and natural preservatives.

Despite the effectiveness of traditional chemical preservatives, the continuous use of these compounds may result in the appearance of resistant strains (EFSA, 2013), and in some toxicity problems (Zengin et al., 2011). Naturally-occurring antimicrobial compounds have particularly attracted much attention as new preservatives, but the number of compounds allowed in foods by legislation is very limited (Weiss et al., 2009). Naturally-occurring antimicrobial compounds include microorganism-based bacteriocins, animal enzymes, polymers or fatty acids, and plant metabolites such as fatty acids or phenolic compounds.

Bacteriocins are antimicrobial peptides produced by some lactic acid bacteria, generally heat stable, apparently hypoallergenic and readily degraded by proteolytic enzymes in the human intestinal tract (Corbo et al., 2009). Bacteriocins can be added to foods as food preservatives or additives ingredients. They can be produced *in situ* by protective cultures, or they can be immobilized in bioactive food packaging (Gálvez et al., 2007). The effectiveness of bacteriocins depends on environmental factors like pH, temperature, food composition and structure, as well as the food microbiota (Gálvez et al., 2007).

Other biomolecules with proved antimicrobial activity are proteins and enzymes, such as lactoferrin and lysozyme, but these hydrophilic compounds are fragile and even small conformational changes can reduce their activity (Dhawan et al., 2018).

Some free fatty acids have the ability to kill or inhibit the growth of microorganisms (Ruiz-Rico et al., 2015). The antimicrobial mechanisms of action are still not completely understood, but the prime target of the free fatty acids seems to be the cell membrane. They disrupt the electron transport chain and oxidative phosphorylation, inhibit enzyme activity, impair nutrient uptake, generate peroxidation and auto-oxidation degradation products or lyse directly the bacterial cells (Desbois & Smith, 2010). The application of free fatty acids on food products has limitations such as the water solubility, the sensory properties that can affect the acceptance of consumers, and the interaction with food constituents that diminishes their antimicrobial activity (Kim & Rhee, 2016).

2.9.1. Essential oils

Essential oils (EOs) are lipophilic extracts of bioactive compounds from aromatic plants, which have displayed antimicrobial activity against several pathogenic and food-borne microorganisms (Langeveld et al., 2014). The inhibitory activity has been attributed to their phenolic compounds and their interaction with microbial cell membranes, which cause leakage of ions and cytoplasmic content, and thus lead to cellular breakdown (Burt, 2004). Despite their remarkable antimicrobial properties, the direct application of EOs in food products has several limitations due to their strong sensory properties (odor and flavor), high volatility, poor solubility and potential toxicity (Hyldgaard et al., 2012). The specifically case of cinnamon essential oil confers an intense odor and flavor, which make it unsuitable for use as a natural preservative on food applications (Dolea et al., 2018). Besides, the concentration of an essential oil needed to inhibit the microbial growth in a food system is higher than in *in vitro* studies, due not only to interactions with food

components (Jo et al., 2015), but also to difficulties in its dispersion in the water phase of food (Weiss et al., 2009).

The mode of action of these natural preservatives has not been fully understood. Considering the different chemical compounds present in EOs, their antibacterial activity cannot be attributable to one specific mechanism having several targets in the cell (Burt, 2004). The essential oil components could act in different ways, including disruption of the cell envelope due to their hydrophobicity that enables them to partition in the lipids of the bacterial cell membrane and mitochondria; disruption of the proton motive force, electron flow and active transport; coagulation of cell contents; and dirsruption of enzymes involved in the energy regulation or synthesis of structural components (Farhan et al., 2018). Extensive studies on the antimicrobial properties of the EOs and their constituents has been carried out and documented by some reviewers (Burt, 2004; Chouhan et al., 2017; Hyldgaard et al., 2012). Herein, some of the most important essential oil components are explained in detail below.

2.9.1.1. Eugenol

Eugenol (4-allyl-2-methoxyphenol) is a hydroxyphenyl propene, naturally occurring in the essential oils of several plants belonging to the *Lamiaceae, Lauraceae, Myrtaceae*, and *Myristicaceae* families. It is largely used in both foods and cosmetics as a flavoring agent. Eugenol presents important biological properties including antioxidant, anti-inflammatory and antimicrobial activities. The antimicrobial effect of eugenol has been demonstrated in studies, being active against fungi and a wide range of Gram-negative and Gram-positive bacteria (Marchese et al., 2017). Eugenol can interfere with the physiology of microorganisms through different mechanisms of action. This compound interferes with membrane functions or suppresses virulence factors, including toxins, enzymes, and the formation of bacterial and fungal biofilm (Burt, 2004; Marchese et al., 2017).

2.9.1.2. Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is obtained from the bean or pod of the tropical vanilla orchid. This phenolic compound exhibits antioxidant and antimicrobial activity against bacteria, mold and yeast (Stroescu et al., 2015). In food industry, this compound may be used as flavor, fragrance and preservative in fruits and vegetables, applied as vapors in storage operations or in modified atmosphere packaging (Fitzgerald et al., 2004). Vanillin is primarily a membrane-active compound, resulting in the dissipation of ion gradients and the inhibition of respiration, the extent to which is species-specific. Vanillin affects membrane integrity by interactions between both lipids and membrane embedded proteins, resulting in leakage of intracellular ions, loss of pH homeostasis and inhibition of key membrane proteins or other cellular functions (Fitzgerald et al., 2004)

2.9.1.3. Thymol and carvacrol

Thymol (2-isopropyl-5-methylphenol) is the main monoterpene phenol occurring in essential oils isolated from plants belonging to the *Lamiaceae* family (*Thymus, Ocimum, Origanum*, and *Monarda*), and other plants such as those belonging to the V*erbenaceae, Scrophulariaceae, Ranunculaceae*, and *Apiaceae* families. This EO is used in the food industry for its flavoring, natural repellent effect and preservative properties (Marchese et al., 2016). Thymol is a bioactive compound with relevant properties as antioxidant, anti-inflammatory, local anesthetic, antiseptic, and especially antibacterial and antifungal (Lambert et al., 2001). Several studies have demonstrated that thymol possesses antibacterial and antifungal activity towards a large range of species encompassing biofilm-embedded microorganisms (Marchese et al., 2016).

The phenolic monoterpene carvacrol (2-methyl-5-(1-methylethyl) phenol) is a major component of the essential oils of *Origanum* and *Thymus* plants (Magi et al., 2015). Similarly to thymol, this molecule presents anti-inflammatory, antioxidant, antitumor, analgesic, anti-hepatotoxic, insecticidal and antimicrobial properties

(Hyldgaard et al., 2012). Carvacrol and thymol are structurally very similar, having the hydroxyl group at a different location on the phenolic ring (Lambert et al., 2001). These compounds are able to disintegrate the outer membrane of microorganisms increasing the permeability of the cytoplasmic membrane to inner components (Burt, 2004). The difference between the antimicrobial efficacy of thymol and carvacrol has been assigned to the hydroxyl group placed at the meta position in thymol, as compared to the ortho position in carvacrol (Pisoschi et al., 2018). It was stipulated that the hydroxyl groups together with the delocalized electron system imparted the powerful antimicrobial effect of carvacrol (Ben-Arfa et al., 2006)

3. Functionalization of supports with antimicrobials compounds

Despite a large number of alternative processing technologies can be found, they present several limitations, including large investment costs, loss of the organoleptic properties, and most important, changes in the nutritional composition of the treated foodstuffs.

Concretely, the filtration technology has been proved to be an excellent alternative, but the materials often have fouling problems and needs to be used in combination or in sequence to achieve a best performance. Fouling caused by rejected colloids, chemicals and microbes, demands considerable attention because it can result in higher energy demand, costly clean-up and frequently replacement of filters (Kim & Van der Bruggen, 2010). The generation of improved safe and stable materials that allow rapid and effective microbial removal is interesting. In this sense, the functionalization of the surface of available filtering materials can be a key approach. For that purpose, the selection of the following parameters is crucial: the adequate material in terms of granulometry and surface features, antimicrobial properties and safety of the immobilized compound, biocompatibility of both support and bioactive molecule, and easy functionalization

procedure. The optimization of these key points could guarantee the correct development of the antimicrobial supports and solve the problems above reported.

Furthermore, the effect of new functionalized materials should be determined not only from the point of view of fouling mitigation and food matrix stability, but also from the point of view of potential (eco)toxicity effects by the possible release of the immobilized molecules to the environment (Kim & Van der Bruggen, 2010). Regarding toxicology and environmental concerns, the best option is the use of extracts naturally present in plants materials as bioactive molecules, because there is evidences that these generate minimum adverse environmental impacts (Hubbe et al. 2015).

The combination of different surfaces with bioactive molecules like naturally-antimicrobial compounds gives improved materials, which offers promising avenues to design effective next-generation supports against bacterial threats using natural compounds (Gupta et al., 2016).

There are different types of supports that could be functionalized with different antimicrobial compounds. The most common materials that could be used as supports to be functionalized and the usual grafting procedures are described in the following subsections.

3.1. Supports

Organic polymeric carriers are the most widely studied materials due to the presence of functional groups, which confer essential interactions with bioactive molecules, have large surface areas, biocompatibility and are easy to manufacture (Udenni Gunathilake et al., 2017). Among natural polymers, cellulose is outlined for its sustainability, renewability, recyclability, wide availability and low cost. Cellulose has no naturally biocide effect, but demonstrates a considerable potential as a solid support or coating to prepare antimicrobial surfaces.

Many current and potential uses of cellulosic materials depend critically on the character of their surfaces. Well-established and emerging strategies to change the outermost surfaces of cellulosic fibers or films not only in terms of chemical composition, but also in terms of outcomes such as wettability, friction, and adhesion, have been developed in the last years (Hubbe et al., 2015).

The surface of cellulosic materials can be modified by heating, by enzymatic treatments, by use of surface-active agents, or by adsorption of polyelectrolytes. The lignin, hemicelluloses and extracts naturally present in plant-based materials also can be expected to play critical roles in emerging strategies to modify the surfaces characteristics of cellulosic fibers with a minimum of adverse environmental impacts (Hubbe et al., 2015). Besides, the chemical modification by coupling agents susceptible to react with the cellulosic matrix constitutes a good way to control the quantity and the nature of the groups present at their surface.

Several types of reagents have been studied in this context, among which anhydrides and isocyanates or organosilanes are the most representative (Abdelmouleh et al., 2002). The chemical grafting of organosilane derivatives is carried out via intermediate silanol groups, which adsorb onto the OH-rich cellulose surface via hydrogen bonding or are subjected to self-condensation, and result in the formation of polysiloxane structures (Salon et al., 2007). The use of organosilanes allows the incorporation of bioactive molecules to the cellulosic surfaces which may confer different physicochemical properties to the cellulose materials.

Inorganic materials, such as silica, are known to be thermally and mechanically stable, non-toxic, and highly resistant to microbial attacks and organic solvents. The surface of siliceous materials presents structural defects in the form of silanol groups (Si-OH), which act as suitable anchoring organic functionalization spots. Accordingly, silanol groups can easily react with organosilane derivatives by means of nucleophilic aliphatic substitution, which results in organic-inorganic hybrid materials. The incorporation of organic moieties allows the precise control of the features of these hybrid materials by considering the chemical nature of the alkyl

chain that contains several reactive organic functional groups, which can be selected to include specific groups in the inorganic framework (Moller & Bein, 1998). Given the large number of available organosilanes, it is possible to develop a wide range of functionalized silica phases with different physicochemical properties by simply varying the organic residue (Hoffmann et al., 2006). However, the number of anchored organic groups is limited by the number of silanol groups on the surface.

3.2. Fundamentals of the immobilization methodology

The modification of material surfaces can be performed according to several immobilization methodologies that are well established. The action mechanisms involve mainly adhesion or coating processes, giving by chemical interaction between the bioactive molecule and the surface. So, it is assumed that adhesion processes are influenced by substances features (Bellmann et al., 2002). Surface grafting offers versatile means to provide existing materials with new functionalities such as hydrophilicity, adhesion, biocompatibility, conductivity, anti-fogging, anti-fouling, and lubrication (Uyama et al., 1998).

Before the immobilization of the bioactive molecule it may be necessary to activate the surface of materials given its inert nature, and therefore, a previous surface functionalization should be carried out by different techniques, such as wet chemical, silane monolayers, UV irradiation, among others (Goddard & Hotchkiss, 2007). Besides the selection of the surface functionalization methodology, the optimization of the technique to introduce the desired type and quantity of reactive functional groups is also needed (Goddard & Hotchkiss, 2007).

The grafting of bioactive molecules can be given by different mechanisms, being the electrostatic linkage and the covalent grafting the most relevant:

a) Electrostatic grafting, taking advantage of the electrostatic interactions between the bioactive molecule and the substrate surface. In some cases, chemical groups are previously anchored to provide an electrostatic charge; with this,

electrostatic interactions ensure a spontaneous grafting of the particles onto the surface. Actually, ionic interactions are also reported to be involved in the strong adsorption of proteins and for biological applications (Girard et al., 2009). An important limitation of physical linkage is the thermal instability due to the relatively weak van der Waals forces or hydrogen bonding that anchors the bioactive compounds to the surface (Mallakpour & Madani, 2015).

b) Covalent grafting, via chemical reaction of the end-functional group with the functional groups on the modified substrate. Covalent surface modification shows greater advantages over physical adsorption because of the enhanced environmental stability (Källrot et al., 2006). Moreover, covalent attachment allows the extension of half-life of the grafted molecule and prevents its release (Goddard & Hotchkiss, 2007).

Covalent attachment of specific biomolecules to surfaces is of great importance for the development of functional supports. The attachment of bioactive molecules to substrates requires an intermediate layer of molecules. A group of molecules that have been widely used are the organosilanes, which participate in the first steps of immobilization procedures for the fabrication of biodevices (Pruna et al., 2016). Organosilanes have unique features, including improved binding affinity, high stability and reduced toxicity (Hu et al., 2017). Thanks to organosilane versatile and exceptional properties, a silane coupling agent often modifies surface properties of solid materials, and assists the linkage between the solid surfaces and functional organics groups, leading to new composites. Making a simple and convenient synthesis of organosilane compounds bearing functional groups could contribute to the development of a wide range of innovative research fields and applications in many industrial sectors (Kobayashi et al., 2018).

The grafting process can be based on the covalent attachment of antimicrobial molecules to the surfaces of the material substrates that bear surface hydroxyls. To this end, hydroxyl moieties react with trialkoxysilane derivatives with a general structure: $(RO)_3Si\text{-}R'$, where R is CH_3- or $CH_3\text{-}CH_2$- and R' is an alkyl chain which contains several reactive organic functional groups (e.g. halogens, amine,

isocyanate, thiol). The presence of such functional groups on the surface of supports allows the direct linking of selected active biomolecules through the formation of covalent bonds.

Other factor to take into account in the grafting of bioactive molecules is the maintenance of the biological properties of the compound after the immobilization. The antimicrobial activity of the natural antimicrobials essential oil components (EOCs) is influenced by their molecular structure. In the molecular conformation, of some EOCs appears a hydroxyl group, which increases the solubility of the compound in aqueous media and improves their ability to pass through the hydrophilic portions of the cell envelope of target bacteria, being recognized as the moiety responsible of the antimicrobial properties. Thus, maintaining the hydroxyl group free is needed. Therefore, Ruiz-Rico et al. (2017) developed an immobilization methodology in which a second reactive moiety capable of reacting with the amine group of alkoxysilane was added to different EOCs by synthesis of aldehyde derivatives of EOCs. Aromatic compounds were derivatized with formylation in ortho position to the hydroxyl group by Vilsmeier and Reimer-Tiemann reactions (Figure 2a). Then, formyl-containing derivatives were grafted onto the external surface of a support previously functionalized with aminopropyl moieties (Figure 2b) (Ruiz-Rico et al., 2017).

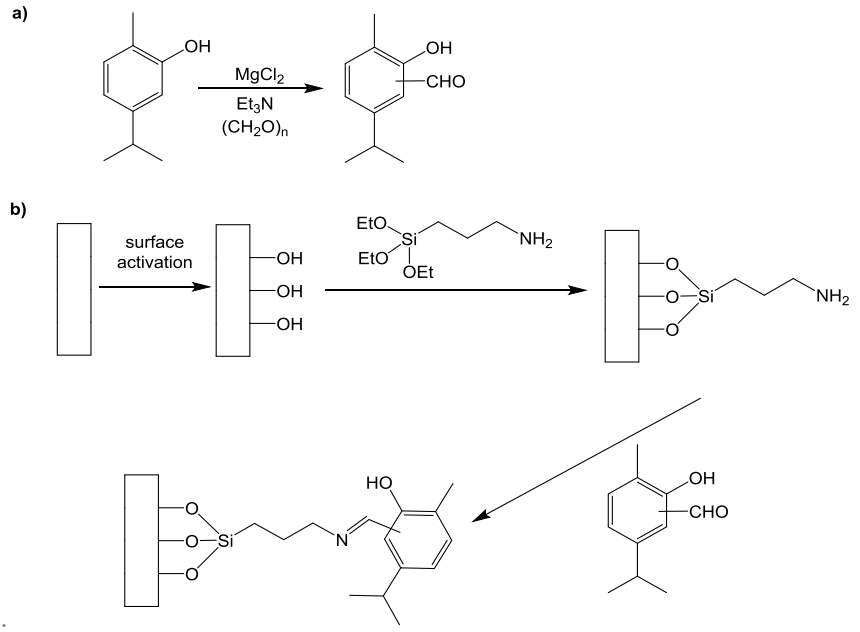

Figure 2. Synthesis procedure of antimicrobial systems functionalized with essential oil components (carvacrol provided as an example). (a) Aldehyde derivatization. (b) Grafting of the derivatized antimicrobial compounds onto the surface of a material functionalized with aminopropyl moieties (Ruiz-Rico et al., 2017).

4. Application of functionalized materials

In recent years, different types of supports functionalized with bioactive molecules have been developed to eliminate microorganisms, but only some of them have been applied as water or food preservation systems, as described below.

Most of the proposed applications based on modified-supports have been used for the design of point-of-use water disinfection systems. Cellulose (commercial filters, absorbent blotting paper and cotton fibers) has been the material par excellence for anchoring silver nanoparticles. In several studies, different immobilization mechanisms have been applied, such as impregnation (Ehdaie et al., 2014; Oyanedel-Craver & Smith, 2008), deposition by *in situ* reduction of silver nitrate on the cellulose fibers (Dankovich & Gray, 2011), or covalent grafting with

previously surface-modified cellulosic supports (Sinclair et al., 2014). In some of them, there is a release of Ag in contact with water; however, the use of cellulosic supports covalently functionalized with Ag nanoparticles prevents the release of the immobilized compound and has shown antimicrobial activity against *Escherichia coli* after filtering contaminated water (Sinclair et al., 2014). Besides, cellulose materials have proved their capability to be electrostatically charged. This modified cellulose was applied in a portable purification system to obtain drinking water. Specifically, softwood pulp fibers and coffee filter paper were functionalized with polyelectrolytes, and the bacteria freely dispersed in water were adsorbed onto the cellulose fibers, which were able to remove more than 99.9% of *E. coli* of water (Ottenhall et al., 2017).

A water filter made of cotton gauzes were coated with chitosan using an UV-curing process or cationized by introduction of quaternary ammonium groups. The materials showed good antibacterial activity against *E. coli*, *Staphylococcus aureus* and *K. pneumoniae* (Ferrero et al., 2014). On a second attempt, water filter for biological disinfection against Gram-positive bacteria was made, resulting in good antibacterial activity (Truffa Giachet et al., 2019). However, a partial release of grafted chitosan was observed, although the water flow reduced the amount of lixiviated chitosan.

As other polymeric material functionalized with bioactive compounds, Cappannella et al., (2016) studied the capability of immobilized lysozyme on chitosan material to preserve wine and avoid the potential allergic reaction derived from the presence of the free enzyme. Lysozyme was covalently immobilized on spherical chitosan supports in order to develop a system for the elimination of lactic bacteria in white and red wine by enzymatic lysis. The particles were placed in a fluidized bed reactor to allow the flow of the substrates, obtaining a high catalytic surface area. Results showed that immobilized lysozyme produced higher lysis of lactic acid than the free form.

As example of other functionalized support, organoclays immobilized onto the surface of sand were developed for the elimination of bacteria in water (Herrera et

al., 2004). The surface of montmorillonite clays was modified by exchanging the predominant interlaminar cations with quaternary ammonium compounds to create organoclays with positive charge able to adsorb and remove bacteria. Microbiological assays showed the antibacterial activity of the composite, eliminating the bacterial load of contaminated water. In another study, the polymeric disinfectant N-halamine siloxane was covalently attached onto silica gel and sand particles. The supports were packed into columns and used as water column filter, resulting in excellent biocidal efficacy against *S. aureus* and *E. coli* (Jiang et al., 2016).

Other siliceous supports like bentonite have been used as material to immobilize Ag and ZnO nanoparticles to develop bentonite chitosan nanocomposites for water disinfection. Nanocomposites were testing on bacteria suspensions at different contact times, resulting in an improvement in the inactivation activity with the introduction of the metallic nanoparticles on the support (Motshekga et al., 2015).

The panorama described above shows that the functionalized supports present a high potential as a water treatment system *in situ*, fast, easy-to-use and low-cost. In addition to this, it is important to highlight the high versatility of these systems, because it can be combined different support materials, different types of compounds to be anchored or grafted, as well as different mechanisms to obtain the functionalized supports. However, so far, industrial applications have not been fully developed because more research is needed on the optimization of these systems to overcome the problems above mentioned. In addition, given the limited research in other types of food, it is interesting to test these supports and/or to develop new ones for other liquid food, such as juices, beers, etc., because the different food matrices could affect the efficacy of these types of systems.

Apart from the works above described, there are some proof-of-concept studies that have demonstrated the antimicrobial efficacy of different combinations of supports–immobilized antimicrobial agent; some of which are shown in Table 1.

Table 1. Supports functionalized with different antimicrobial compounds.

Support	Immobilized antimicrobial agent	Study	Reference
Cellulose acetate nanofibrous mats	Lysozyme and *N*-[(2-hydroxy-3-trimethyl-ammonium) propyl] chitosan chloride	Antimicrobial activity against *E. coli* and *S. aureus* by immersion in diluted bacteria suspension.	(Huang et al., 2013)
Chitosan beads	Lysozyme	Antimicrobial activity against *Oenococcus oeni* in white wine by measurement of turbidity.	(Liburdi et al., 2016)
Halloysite	ZnO and CeO_2 nanoparticles	Nanocomposites was re-suspended in the test tube with *E.coli* and then the antibacterial activity was measured by standard plate count method.	(Shu et al., 2017)
Cellulose acetate nanofiber	Ag NPs on TiO_2 and CA/TiO_2/Ag NPs	Inhibition zones by disk diffusion tests to determine antimicrobial activity of nanocomposite particles and nanofibers against *E. coli* and *S. aureus*.	(Jatoi et al., 2019)
Surface-grafted chitosan	Lysozyme	Antibacterial test against *S. aureus* by *in vitro* study.	(Yuan et al., 2013)
Cellulose nanofibers	Ag nanoparticles	*In vitro* antimicrobial activity against *E. coli*	(Lala et al., 2007)
Mesoporous silica nanoparticles	Lysozyme	Development of antimicrobial agents against *E. coli* evaluated *in vitro* and *in vivo* for biomedical applications.	(Li & Wang, 2013)
Nanostructured cellulose membranes	N-(3-trimethoxysilylpropyl) diethylenetriamine (N3)	Antimicrobial activity against *S. aureus* and *E. coli* in *in vitro* studies for biomedical applications.	(Fernandes et al., 2013)
Cellulose nanofiber composites	Ag nanoparticles	Antimicrobial activity against *S. aureus* and *E. coli* in *in vitro* studies.	(Gopiraman et al., 2016)
Fumed silica, amorphous silica and mesoporous silica microparticles	Carvacrol, eugenol, thymol and vanillin	Development of antimicrobial agents against *S. aureus, L innocua* and *E. coli* evaluated *in vitro* and *in situ* (preservative additive in milk).	(Ruiz-Rico et al., 2017)
Mesoporous silica microparticles	Eugenol and thymol	*In vitro* and *in situ* (strawberry jam) of novel food preservatives against spoilage mold and yeast.	(Ribes et al., 2017)

Support	**Immobilized antimicrobial agent**	**Study**	**Reference**
Microfibrillated cellulose	β-lactam antibiotic benzyl penicillin	Contact active antimicrobial films against *E. coli* and *S. aureus* for food or medical applications.	(Saini et al., 2015)
Cellulose nanofibers	Amino silanes	Contact active antimicrobial materials against *Bacillus subtilis, S. aureus* and *E. coli.*	(Saini et al., 2017)
Silica particles	Cationic amphiphilic cyclic carbonates	*In vitro* antimicrobial activity of the functionalized particles against *S. aureus*, *E. coli* and *P. aeruginosa.*	(Xu et al., 2016)
Silica nanoparticles	Gentamicin, neomycin and kanamycin.	*In vitro* antimicrobial activity against clinical pathogens: *Bacillus cereus*, *S. aureus*, *E. coli* and *Salmonella enterica typhimurium* for biomedical applications.	(Agnihotri et al., 2015)
Polyamide thin film composite membranes	Ag nanoparticles-coated silica particles	Antimicrobial membranes against *E. coli, Pseudomonas aeruginosa* and *S. aureus* in *in vitro* studies.	(Park et al., 2016)
Silica nanoparticles	Quaternary ammonium silane	Antimicrobial functionalized nanoparticles against *E. coli*, *S. aureus and Deinococcus geothermalis*.	(Song et al., 2011)
PET films and filter paper	Pyridinium groups	Development of antimicrobial surfaces against *E. coli* by airborne or waterborne antibacterial assays.	(Cen et al., 2003)
Silica micro beads	Ag nanoparticles	Development of antimicrobial functionalized silica beads *against E. coli* and *B. subtilis* by zone inhibition and test tube tests.	(Quang et al., 2011)

5. Conclusions and future perspectives

Different non-thermal preservation technologies for water and liquid food are being used to assure their stability and safety. These processes are efficient preservation methods but can generate some changes in nutritional, structural and organoleptic food properties, and imply high investment and production costs. In addition, in some cases, toxic compounds can be generated, such as in some water disinfection processes.

The use of traditional antimicrobial agents has been successfully implemented for many years, but their negative impact on human health has become imperative to create alternatives. In this sense, some naturally-occurring compounds, such as essential oils, have been reported to exhibit high antimicrobial activity, but the incorporation of these natural preservatives in foods also produce changes in the organoleptic properties because of their strong aroma and flavor. In order to compensate the essentials oils limitations, their immobilization onto the surface of organic and inorganic supports has been recently developed, obtaining remarkable antimicrobial properties of the functionalized supports.

Moreover, filtration systems could be an interesting alternative in addition to their common use in the food industry for clarification, concentration and microbial stabilization. Recently, alternative materials have been proposed in order to create filtration systems as preservation technology. Filtration systems have been made of many filtering materials, but the cellulose and the silica particles have interesting physical and chemical properties that give them advantages over other materials. The preservation mechanism due to immobilization of antimicrobial agents on the supports has great potential applications, especially in water treatment, as reported before. However, the immobilization techniques should ensure the capability of the filter to remove the microbial load without affecting the features of the treated liquid food and preventing the leaching of the immobilized compounds. In this context, the development of new preservation technologies that meet all the requirements previously mentioned is needed. This certainly opens a way of study in a field little explored in the food preservation technology.

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3. OBJECTIVES

3. OBJECTIVES

The main objective of this PhD thesis was to develop and apply filtering materials for the microbial reduction on liquid food.

To achieve the main objective, two specific objectives were proposed:

a. **To study the development of cellulosic filtering materials for the treatment of liquid food**.

   I. To synthesize, characterize and apply microparticulated cellulose supports as filtering materials for beverages.

   II. To synthesize, characterize and apply cellulose membranes functionalized with polyamines as filtering materials for water.

   III. To assess the effect of the filtering cellulosic supports on the microbiological quality of the liquid food.

b. **To study the development of functionalized silica supports for filtering liquid food.**

   IV. To synthesize, characterize and apply silica supports functionalized with essential oil components as filtering materials for beverages.

   V. To assess the influence of the filtering treatment on the physicochemical, microbiological and sensory parameters of the food matrix.

   VI. To determine the potential leaching of the immobilized bioactive compounds to treated liquid foods.

4. EXPERIMENTAL APPROACH

4. EXPERIMENTAL APPROACH

The present doctoral thesis is structured in two chapters, which includes articles published or that are under revision in international scientific journals. In order to give an overview of the experimental work that has been carried out, this section details the methodology used in each chapter.

Chapter I consisted of the development of cellulosic materials to filter water and fruit juice (Figure 1). This objective is addressed in Articles 1 and 2. The Article 1 represents a first approach to develop a filtering aid using a by-product of corn production (native corn stalks), which was delignified to obtain a tubular cellulose filtering material. After the synthesis and characterization processes, a previous optimization study was carried out with water contaminated with *Lactobacillus casei* and *Saccharomyces cerevisiae*. Once the best filtering conditions were obtained, two continued pasteurization tests were carried out. The developed material was placed in a cylindrical bioreactor to form a well packed filter column. Subsequently, water and orange juice contaminated with *S. cerevisiae* were filtered in a continuous flow for 10 and 20 days, respectively.

In Article 2 a study was performed to improve the retention capacity of the cellulose material. In this work, cellulose membranes were functionalized with an organosilane for the covalent immobilization of polyamine onto the paper membranes. The removal properties of the amine-functionalized membranes were assayed in different filtration assays to treat water inoculated with *E. coli.*

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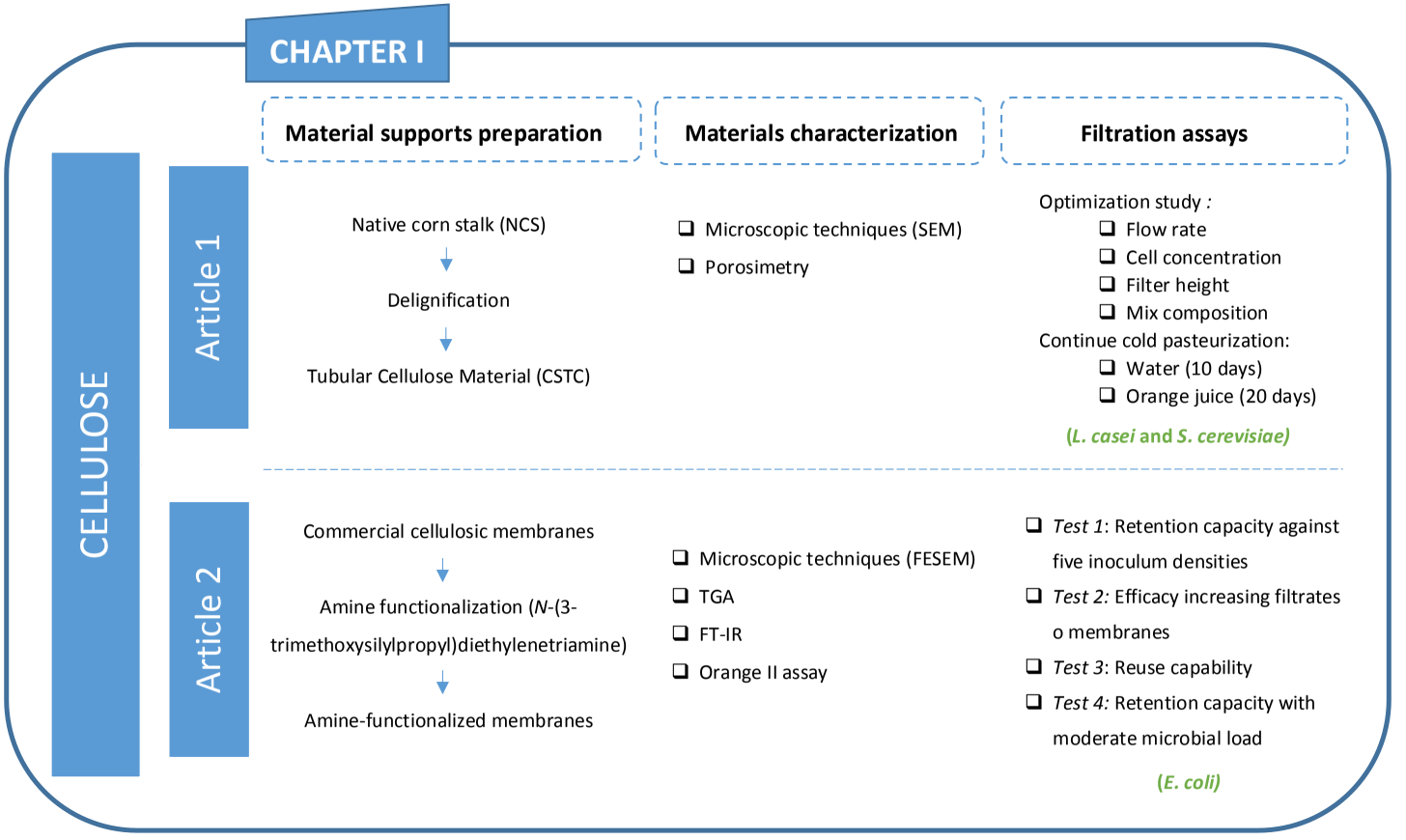

Figure 1. General scheme of the experimental work of the chapter I.

The **Chapter II** focuses on the development of filtering materials based on silica supports for the treatment of liquid food. For the synthesis of the functionalized supports, essential oil components were covalently grafted onto the surface of silica microparticles with different mean particle size by using organosilanes as coupling agents (Figure 2). After characterization of the supports, the functionalized silica microparticles were used as filtering aids for bed filtering of liquid food to remove both pathogenic and spoilage microorganisms. In Articles 3 and 4 the application of the developed functionalized silica supports is studied by different filtration assays, using water and beer as food matrices. Finally, the Article 5 evaluated the application of some of the best supports in the treatment of fruit juice with an additional approach, which was the evaluation of the effect of the filtering system on the physicochemical, microbial and sensory food properties and shelf life of the product. In parallel to the microbial removal studies, the possible mechanism of action of the functionalized supports was evaluated by means of microscopic and plate count techniques. In addition, the possible leaching of the immobilized essential oil components was determined by analyzing the filtered fluid (water, beer and apple juice) by instrumental techniques.

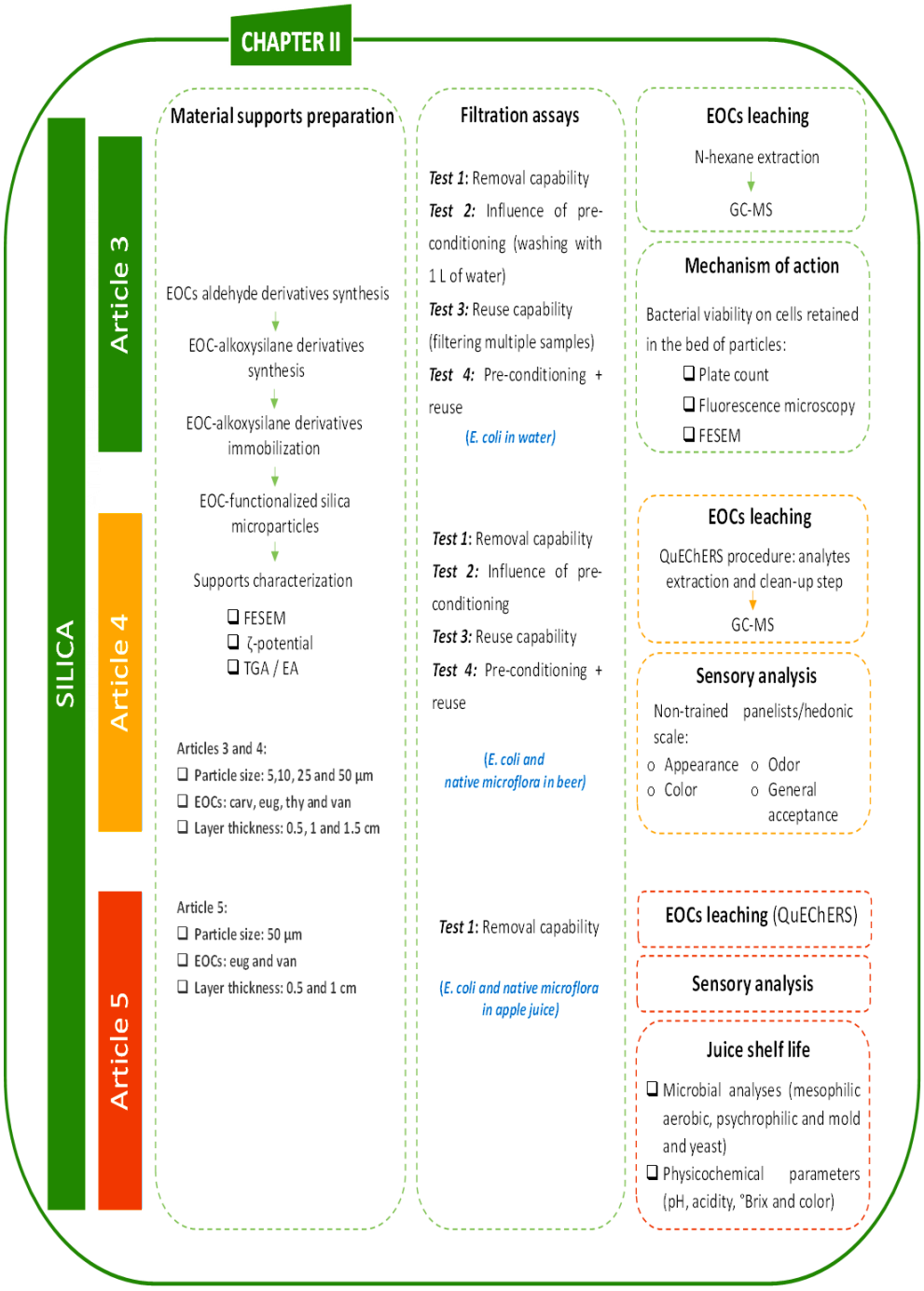

Figure 2. General scheme of the experimental work of the chapter II.

5. CHAPTER 1. DEVELOPMENT OF CELLULOSIC FILTERING MATERIALS

5.1. Cold pasteurization of liquid food using tubular cellulose filter from corn stalks

Nataly Peña-Gómez[1], Vasilis Panagopoulos[2], Maria Kanellaki[2], Athanasio A. Koutinas[2], Maria Ruiz-Rico[1], Isabel Fernández-Segovia[1], José M. Barat[1]

[1]Grupo de Investigación e Innovación Alimentaria, Departamento de Tecnología de Alimentos, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[2]Food Biotechnology Group, Department of Chemistry, University of Patras, 26500 Patras, Greece. Tel.:0030.2610.997104 e-mail: A.A.Koutinas@upatras.gr

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Abstract

An attractive cold pasteurization technology based on nano/micro-porous cellulosic material (or tubular cellulose, TC) from native corn stalks (NCS), is presented in this study. The aim of this investigation was to develop a non-thermal system for microbial stabilization of water and orange juice by a continuous flow system, which must be able to keep the original organoleptic characteristics, environmentally acceptable and cost effective. In this sense, corn stalks were selected because they are composed by a lignocellulosic material with high porosity and are available in large quantities. After an optimization study of NCS and corn stalk tubular cellulose material (CSTC) to remove yeast *Saccharomyces cerevisiae* and *Lactobacillus casei* from water, the continuous cold pasteurization of commercial orange juice was performed for 20 days to remove *S. cerevisiae*, and organoleptic properties of the juice were evaluated. The results showed that the delignified CSTC was found effective as filter for the removal of *S. cerevisiae* and *L. casei* from water. CSTC was a successful filtering material for the treatment of commercial orange juice contaminated with yeast cells in a continuous mode. In addition, the color properties only were affected while filter regeneration was performed. The proposed process is a low-cost and promising alternative to existing thermal pasteurization technologies.

Keywords: Cold pasteurization; water, orange juice; tubular cellulose; corn stalks; *S. cerevisiae*; *L. casei.*

1. Introduction

In 2016, European corn production area covered approximately 18 million hectares (FAO, 2018). For every 100 kg of corn grain, 18 kg of corn stalks are produced (Tsai et al., 2001), doing the corn stalk core the most abundant crop residue accounting almost 50% of the total agricultural waste production (Vafakhah, Bahrololoom, Bazarganlari, & Saeedikhani, 2014; Zheng, Dang, Yi, & Zhang, 2010). Such a large quantity poses a serious problem not only in storage, but also in waste disposal (Tsai et al., 2001), creating significant environmental problems. In order to face disposal problem, researchers are continually trying to find alternative solutions. Therefore, corn stalk core is proposed to be used as an animal feed (Mani, 2006), as raw material for production of biofibers (Reddy & Yang, 2005), biomass for ethanol production (Sassner, Galbe, & Zacchi, 2008) and starting material for the preparation of active carbon compounds (Cao et al., 2017; Ioannidou, Zabaniotou, Stavropoulos, Islam, & Albanis, 2010).

Approximately, 50 percent of the corn stover is the stalk that mainly consists of cellulose, hemi-celullose and lignin. Until recently, few studies have been carried out concerning the handling or reuse of corncob wastes (Lu & Chen, 2014; Lv et al., 2013). Considerations for other potential uses are in progress based on the material's adsorbent and densification properties (Kaliyan & Morey, 2010; Nkayem, Mbey, Diffo, & Njopwouo, 2016; Suteu, Malutan, & Bilba, 2011). Furthermore, due to their porous and tubular structure (Vafakhah et al., 2014), corn stalks could be great candidates to be used as filtering materials for liquid food preservation. Ideally, not only should a food preservation method keep the original organoleptic characteristics and improve shelf-life, but also should be easily applicable, environmentally acceptable and cost effective (Gialleli, Kallis, Bekatorou, Kanellaki, & Koutinas, 2015).

In fruit juices the principal spoilage is due to microbial growth of yeasts and molds, such as *Saccharomyces cerevisiae* (Velazquez-Estrada, Hernandez-Herrero, Guamis-Lopez, & Roig-Sagues, 2012), as well as certain acid-tolerant bacteria like *Lactococcus, Lactobacillus*, and *Leuconostoc* (Guerrouj, Sanchez-Rubio, Taboada-

Rodriguez, Cava-Rolla, & Marin-Iniesta, 2016; Hodgins, Mittal, & Griffiths, 2002). Orange juice spoilage, in particular is prevented by pasteurization (HTST- High Temperature Short Time) (Lee & Coates, 2003).

Lately, researchers have proposed several mild preservation technologies for orange juice processing, such as high pressure pretreatment (Bull, 2004; Evelyn & Silva, 2016), ultra-high pressure homogenization (Velazquez-Estrada et al., 2012), high pressure carbon dioxide processing (Briongos et al., 2016), UV irradiation treatment (Rodriguez, Oteiza, Giannuzzi, & Zaritzky, 2017; Taze, Unluturk, Buzrul, & Alpas, 2015), ultrasound and microwave application (Samani, Khoshtaghaza, Lorigooini, Minaei, & Zareiforoush, 2015) and thermal enzyme inactivation (Aghajanzadeh, Ziaiifar, Kashaninejad, Maghsoudlou, & Esmailzadeh, 2016). However, these preservation methodologies encounter problems like cavitation effect, micro-mechanical shocks, lower decimal reduction of bacterial and mold spores and changes on sensory characteristics of citrus juices (Evelyn & Silva, 2016).

Previous studies have assessed the effectiveness of tubular cellulose (TC) as filter filling materials for cold pasteurization of wine (Gialleli et al., 2014), water (Kumar, Gialleli, Bekatorou, Koutinas, & Kanellaki, 2016) and apple juice (Gialleli, Bekatorou, Kanellaki, Nigam, & Koutinas, 2016) through filtration. The aim of this investigation was the development of a novel technology for massive employment of the porous corn stalks, which enabled the reduction of environmental pollution and further created added value in corn production. Therefore, in order to develop an attractive cold pasteurization technology based on corn stalks, the removal of microorganisms *S. cerevisiae* and *L. casei* from the water and orange juice in a continuous flow system has been investigated.

2. Materials and Methods

2.1. Corn stalks core material support

The corn stalks were provided from a crop corn field situated in Alfiousa (Pyrgos, Greece) village. The corn stalks were cut into pieces of (2x2cm) and were washed several times with hot water (70–80 °C) prior to use (Native Corn Stalks-NCS).

2.2. Delignification of corn stalks core material

In order to obtain corn stalk tubular cellulose (CSTC) material, the native corn stalks (NCS) were cut in smaller pieces and then a quantity of 300 g was treated with 3 L NaOH 1% for 3 h as described by Papafotopoulou-Patrinou et al. (2015).

2.3. Microbial strains and media

In order to study the removal of microbial cells by filtration through a corn stalks tubular filter, the alcohol-resistant and psychrotolerant yeast strain *Saccharomyces cerevisiae* AXAZ-1 and the lactic acid bacteria *Lactobacillus casei* ATCC 393 (DSMZ, Germany) were used. *S. cerevisiae* was grown in a synthetic medium containing 1 g NH_4SO_4, 1g KH_2PO_4, 5 g $MgSO_4$, 40 g glucose, and 4 g yeast extract. *L. casei* was grown at 37 °C for 72 h in MRS Broth (Merck, Darmstadt, Germany) under anaerobic conditions. *L. casei* cells were harvested by centrifugation at 4125 g for 10 min at 20 °C (SIGMA 3 K12, Bioblock Scientific, Laborzentrifugen GmbH, Osterode, Germany). Commercial clarified orange juice without pulp was used in the experiments (pH 4.04; total sugars 90 g/L; no preservatives).

2.4. CSTC filter preparation

The filling materials produced according to sections 2.1 and 2.2 were tightly wrapped in a perforated nylon fabric to create a cylindrical filter, which was then

placed in a cylindrical glass bioreactor to form a well packed column (Figure 1). Two filters with different sizes with a height of 42 and 50 cm were produced.

2.5. Scanning electron microscope

The filling materials were characterized using standard instrumental techniques. A morphological analysis of tubular cellulose microstructure was performed by scanning electron microscope (SEM) model JSM-6300 (Jeol, USA). Samples were dried overnight at 40 °C and then were coated with gold in a Balzers Sputter Coater SCD 004 (Balzers, Switzerland) for 3 min before analysis.

2.6. Porosimetry analysis

The NCS and CSTC materials were grinded to obtain dust particles in order to carry out the porosimetry analysis for determination of the average pore diameter and pore volume of NCS and TC, according to the method proposed by Koutinas et al. (2016). The N_2 physisorption test was carried out at −196.15 °C on a Tristar 3000 porosimeter (Micromeritics) after degassing at 100 °C for 2 h. The BET (Brunauer, Emmett and Teller) specific surface area was determined. Finally, pore volume and average pore diameter were calculated using the model of Barret, Joyner and Halenda (BJH).

2.7. Optimization study of cold pasteurization

2.7.1. Filtration assembly design

The system consisted of a (1) cylindrical glass bioreactor of 2 L (70 cm; 5 cm i.d.) packed with the CSTC filter, (2) a high accuracy peristaltic pump (MINIPULS Evolution® Peristaltic Pump from Gilson, France) and (3) a magnetic stirrer for the inflow (Fig. 1).

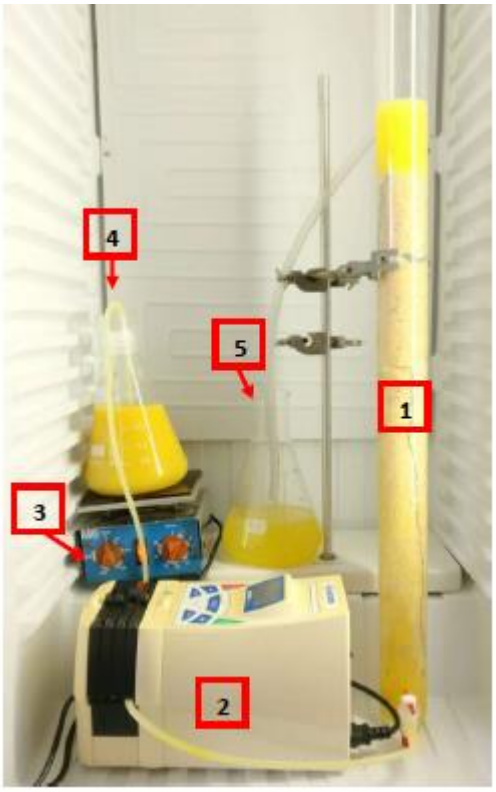

Figure 1. Cold pasteurization system based on a packed-bed bioreactor with corn stalks tubular-cellulose (CSTC) particles. (1) filter, (2) peristaltic pump, (3) magnetic stirrer, (4) system inlet, (5) system outlet.

2.7.2. Effect of cell concentration and flow rate

The effect of different concentrations of *S. cerevisiae* and *L. casei* microbial cells and upstream flow rate on the microbial removal was analyzed in the two types of corn stalks core materials (NCS and CSTC). Those assays were carried out in order to select the best material and determine the most effective operating conditions for the filtration system.

Initially, the continuous system was supplied successively with water contaminated with *S. cerevisiae and L. casei* separately and in mixtures, using filters of different height (42 and 50 cm). Likewise, delignified (CSTC) and non-delignified

corn stalks (NCS) were used for the filters production. In the frame of this differentiation, the effects of initial cell concentration of contaminated water and the flow rate were examined by four successive tests (Table 1). Test 1 studied the effectiveness of both materials in function of the cell concentration in a constant flow rate (1 L/day). Once the best material was selected, this material was evaluated in two bioreactors with different filter height (42 and 50 cm) to establish the effect of cell concentration (Test 2) and determine the flow rate (Test 3). Finally, once the best filter height was selected, this bioreactor was probed in different flow rate and mixtures of both microorganisms (Test 4).

For each assay, the tested filter (NCS and CSTC) was freshly produced and dried. Then the contaminated water was pumped through the vertical glass cylinder that was filled with the tested filter by using the peristaltic pump. Samples were collected from the outlet stream for cell concentration determination. After complete passage of the liquid through the filter, the vertical glass cylinder was emptied, the remaining liquid was discarded and new contaminated water was pumped, for another measurement. Each time a contaminated liquid passed through the filter or a change in one parameter took place, the filter was washed with hot water (70–80 °C).

Table 1. Parameters evaluated on the different water filtration tests.

Test 1.		**Test 2.**		**Test 3.**		**Test 4.**		
Cell concentration (g/L)		**Cell concentration (g/L)**		**Flow rate (L/day)**		**Mix composition (%)**		**Flow rate (L/day)**
S.c	*L.c*	*S.c*	*L.c*	*S.c*	*L.c*	*S.c*	*L.c*	-
0.5	0.3	0.5	0.1	0.5	0.3	50	50	0.5
1	0.5	1	0.3	0.7	0.5	66.66	33.33	0.7
1.5	0.7	1.5	0.5	1.5	0.7	76.92	23.08	1
2	1	2	0.7	2				
			1					

S.c: Saccharomyces cerevisiae and *L.c: Lactobacillus casei*

2.8. Cold pasteurization of water and orange juice

Water and the orange juice were contaminated with 2 g/L (7 log CFU/mL) of *S. cerevisiae* and were filtered through CSTC support with a continue upstream flow rate of 1.5 L/day. The effectiveness of the pasteurization technique was evaluated by standard plate counting of microbial population of *S. cerevisiae* of the inlet and outlet liquid stream. When the microbial removal efficiency was reduced below 70%, the regeneration of the filter took place by washing with previously sterilized hot water (70–80 °C). More specifically, water was pumped upstream through CSTC of corn stalks using a flow rate of 200 mL/min and measuring the optical density of the outflow until absorbance values of zero when the regeneration was considered to be completed and the cold pasteurization process continued. The operational stability of both systems was monitored for 10 and 20 days in water and orange juice, respectively.

2.9. Cell concentration determination

The removal percentage was determined by plate counting and optical measurements of the samples before and after the filtration thought the CSTC. Viable counts of *S. cerevisiae* were enumerated in triplicate after plating 0.1 mL of appropriate dilutions on Potato Dextrose Agar (Fluka) and incubating the plates at 30 °C for 72 h. The count in the samples was expressed as log CFU per milliliter. The optical density was measured at wavelength of 700 nm in Jenway 6300 UV/VIS spectrophotometer (Staffordshire, UK) with calibration curves.

2.10. Color index of orange juice

The color index (absorbance at 420 nm) was measured using a Jenway 6300 UV/VIS spectrophotometer analysis. The absorbance was determined according to the Cortes, Esteve, and Frigola (2007) method with modifications. Three milliliters of orange juice were centrifuged at 2000 *g* for 20 min at 18 °C, and the supernatant absorbance was measured at 420 nm.

2.11. Statistics

All analyses were carried out in triplicate (n=3) and the results are presented as mean values and standard deviations. The results were analysed by one-way and multifactor analysis of variance (ANOVA) to evaluate the effect of the factor on the microbial reduction. The least significance procedure (LSD) was used to test for differences between averages at the 5% significance level. Data were statistically processed by the Statgraphics Centurion XVI software.

3. Results and Discussion

3.1. Rational of the investigation

This study attempts to develop a simple low-cost, easy scaling up of production and lower energy consumption cold pasteurization technique using corn stalk as a novel filtration filling, that is an abundant, renewable and fully biodegradable material. Thus, it is possible to generate alternatives to solve big problems, avoiding the undesirable quality effects of conventional high-temperature processing and the high cost of non-thermal and membrane technologies, and finally offer an alternative use of the largest crop residue of the agricultural production. Corn stalks were selected as starting materials for the development of filtering supports because it is a lignocellulosic material with high porosity and is available and concentrate in large quantities in corn production plants. Therefore, this appears to be an ideal lignocellulosic raw material for the development of suitable filter for microbial cells removal through their entrapment in corn stalks tubes. In order to increase the number and size of the cellular tubes of corn stalks, removal of lignin was performed in the present study.

A previous study used wood sawdust to produce porous cellulose filtering material (Gialleli et al., 2014). However, in this study we used other naturally-occurring materials with better properties like corn stalks because of their low density being much lighter materials, their abundance as agricultural waste product

and their negligible cost. This lighter material enables easier handling of larger volumes for production of industrial filters compared to wood.

In the current study, several parameters were evaluated for assessing the optimal conditions of the developed filters, including (i) the type of filtering material (NCS versus CSTC), (ii) the length of the filter and (iii) the effect of initial microbial cell concentration and flow rate of water contaminated with yeast and bacteria. *S. cerevisiae* cells were selected because yeast are the most abundant microorganisms that spoil juices having low pH, while *L. casei* was used as representative of lactic acid bacteria that may be found in citrus juices. Furthermore, the cell size of the two microorganisms differs significantly. After the optimization study, a continuous cold pasteurization of water and commercial orange juice was performed for 20 and 11 days, respectively, using two separate bioreactors.

3.2. Material characterization

Material characterization was carried out by porosimetry analysis to establish the surface area of the particles and pore sizes. Surface area is an important parameter affecting the maximum adsorption capability and thus the hydrolysis of cellulose (Ye & Berson, 2014). The results of BET analysis (Table 2) indicated that after delignification process there is a slight increase in total specific surface area. Similar observations has been reported for alkaline pretreated corn stalk (Kandylis, 2018). Table 2 also presents the surface area, as well as the pore volume and pore size for non-treated and delignified corn stalk. An increment of the total specific surface area is observed while the size of the pores between 1.7 and 300 nm decreases. This can be attributed to the formation of pores in the region below 1.7 nm. The increased enzymatic hydrolysis rate, which took place in the corn stalk delignification process (Kandilys et al., 2018), have correlated with corresponding increase of available surface area using different cellulose and lignocellulosic substrates (Sinitsyn, Gusakov, & Vlasenko, 1991) as well as with the decrease of the

pore size. The results showed lower pore size compared to other natural porous materials, whose values were found between 15 and 17 nm for delignified sawdust obtained from sal wood and mango wood, respectively. Indeed the CSTC and NCS materials had a pore size with values close to delignified rice husk and pine sawdust delignified materials (Koutinas et al., 2012; Kumar et al., 2014).

Additionally the formation of smaller size pores would be great advantage for efficient microbial removal while the microbial cells with size smaller than 1.7 nm can be retained by those pores.

Table 2. Porosimetry results of native corn stalk (NCS) and corn stalk tubular-cellulose (CSTC).

Treatment	BET surface (m^2/g)	Macro-, mesopore (1.7-300 nm)		
		$A_{BJH}(m^2/g)$	$V_{BJH}(cm^3/g)$	Pore size (nm)
CSTC	0.846±0.011	0.57	0.0044	7.36
NCS	0.808±0.003	0.75	0.0049	9.02

3.3. Optimization study

The effect of cell concentration on the microbial removal capability of the filters was carried out with two types of filling materials (NCS and CSTC) in order to select the most efficient one. Figure 2 shows the percentage of cell removal of the filters according to the cell concentration of the tested microorganisms in water. As can be seen, microbial concentration played a crucial role on the microbial load removal efficiency. The results showed that at higher concentrations of *S. cerevisiae* (2 g/L) and *L. casei* (1 g/L) cells, CSTC was more efficient than NCS. In addition, the Figure 3 shows an opposite behavior for each material in *L. casei* treatment. When cell concentrations increased, in the case of NCS the removal capacity is reduced, but CSTC can be continue effective. In general, CSTC was more effective in all cases than NCS for any concentration.

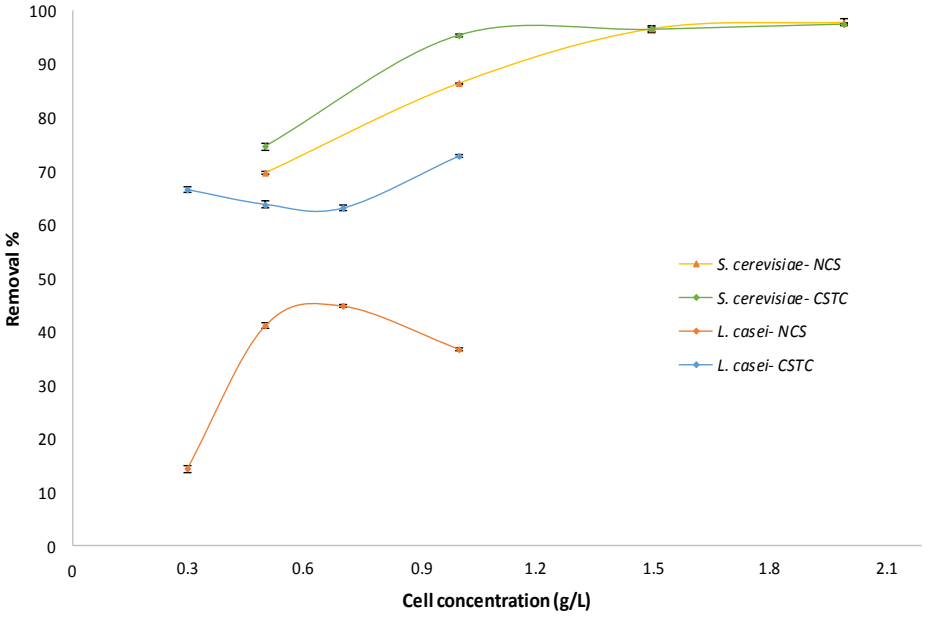

Figure 2. Percentage of cell removal of *S. cerevisiae* and *L. casei* cells at different concentrations in water using native corn stalk (NCS) and corn stalk tubular-cellulose (CSTC) filters in a flow rate of 1 L/day through a 50 cm height filter (means and standard deviations, n=3).

SEM images of filtering materials after filtering samples showed attachment and entrapment of both *S. cerevisiae* (Fig. 3a) and *L. casei* (Fig. 3c) cells in the pores of the native corn stalk material, but the amount of retained cells was clearly higher on CSTC material (Fig. 3b).

The SEM results agree with those reported by Kourkoutas, Bekatorou, Banat, Marchant, and Koutinas (2004). Cell attachment on CSTC could be due to physical adsorption by hydrogen bonding, other weak forces, as well as natural entrapment in the CSTC tubes of the material (Koutinas et al., 2012). Accordingly, CSTC material was selected to continue with the assays for determination of the optimum operating conditions of the filtration system for cold pasteurization.

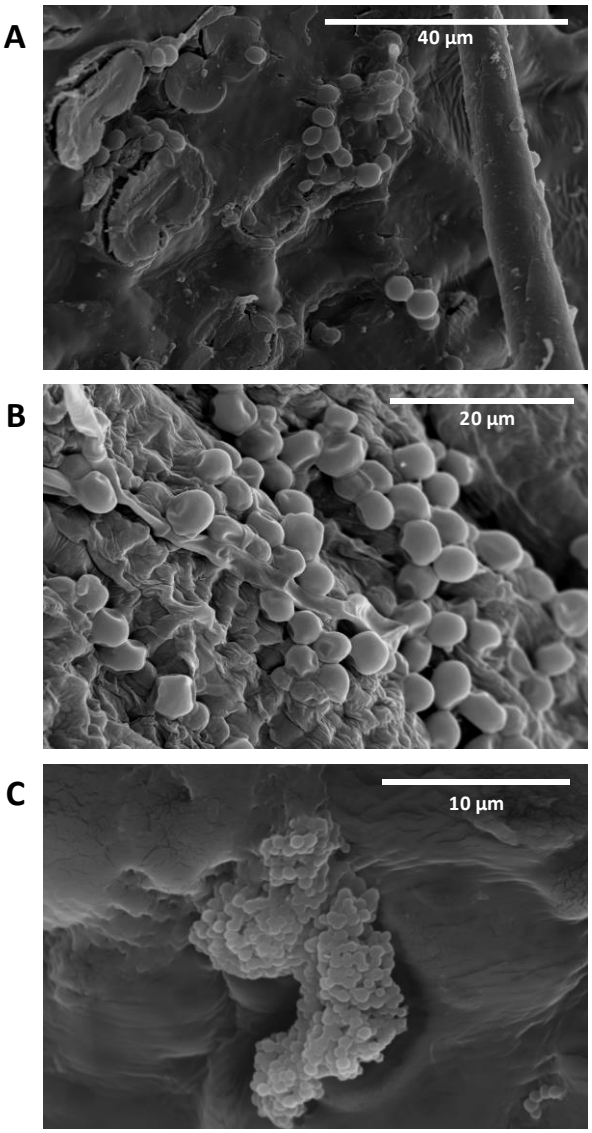

Figure 3. SEM images of the filters with entrapped *S. cerevisiae* in native corn stalk (NCS) (A), and corn stalk tubular cellulose (CSTC) (B) and entrapped *L. casei* in native corn stalk (NCS) (C).

Once the CSTC material was chosen, two different height (42 and 50 cm) of the CSTC filter, different cell concentrations (Test 2) and flow rate (Test 3) were evaluated to establish their influence on the removal of *S. cerevisiae* and *L. casei*. Table 3 shows the percentage of cell removal of both microorganisms according to the cell concentration and filter height with a flow rate of 1 L/day. The results confirmed that the initial cell concentration is directly related to the cell removal capacity, which implies that higher concentrations result in higher percentage of removal and that filters are more efficient against *S. cerevisiae* compared with *L. casei.* This could be explained by the different size of cells, the bigger yeast cell can be retained more easily. In this sense, a concentration of 1 g/L of *S. cerevisiae* and *L. casei* was chosen for development Test 3.

Table 3. Effect of cell concentration on the removal of *S. cerevisiae* and *L. casei* cells in water using corn stalk tubular-cellulose (CSTC) filter, in a flow rate of 1 L/day. Mean ± Standard deviation (n=3).

	S. cerevisiae		***L. casei***	
Filter Height (cm)	**Inlet cell concentration (g/L)**	**% Cell removal**	**Inlet cell concentration (g/L)**	**% Cell removal**
42	0.5	74.76±0.95^{a}	0.1	44.60±1.05^{a}
	1	95.47±0.91^{b}	0.3	44.42±1.02^{a}
	1.5	94.03±1.00^{b}	0.5	69.18±1.02^{b}
	2	96.53±0.49^{b}	0.7	68.55±0.67^{b}
	-	-	1	45.42±1.12^{a}
50	0.5	71.18±0.89^{a}	0.1	51.54±0.54^{a}
	1	94.90±0.37^{b}	0.3	63.55±0.51^{c}
	1.5	96.89±0.73^{c}	0.5	60.39±0.26^{b}
	2	97.80±1.00^{c}	0.7	61.24±0.71^{b}
	-	-	1	69.76±0.68^{d}

Table 4 shows the percentage of cell removal of both microorganisms according to the flow rate and filter height with an inlet cell concentration of 1 g/L. Test 3

demonstrated that the flow rate had a different effect on each microorganism (Table 4). In case of *S. cerevisiae*, the increased flow rate of the system improve the cell retention capacity. Bye other way, the removal capacity of *L. casei* required a reduction of flow rate in the filtration system.

However, the removal capacity of *L. casei* is significative effected ($p<0.05$) by the filters height. Although no important differences were observed between filters of 42 or 50 cm for *S. cerevisiae* treatment.

In general, higher height of filter encourage the microbial retention capacity of CSTC and the flow rate of system have a significant effect ($p<0.05$) on the filtration effectiveness in both cases.

Table 4. Effect of flow rate on removal of *S. cerevisiae* and *L. casei* cells in water with initial concentration 1 g/L, using a corn stalk tubular-cellulose (CSTC) filter. Mean ± Standard deviation (n=3).

	S. cerevisiae		***L. casei***	
Filter Height (cm)	**Flow rate (L/day)**	**% Cell removal**	**Flow rate (L/day)**	**% Cell removal**
42	0.5	93.12 ± 0.51^{b}	0.3	84.50 ± 0.69^{c}
	0.7	95.44 ± 0.46^{c}	0.5	69.71 ± 0.58^{b}
	1.5	95.02 ± 0.60^{c}	0.7	54.95 ± 0.74^{a}
	2	85.67 ± 0.78^{a}	-	-
50	0.5	95.62 ± 0.26^{a}	0.3	90.28 ± 0.84^{c}
	0.7	95.69 ± 0.26^{a}	0.5	84.73 ± 0.20^{b}
	1.5	95.72 ± 0.19^{a}	0.7	75.53 ± 0.51^{a}
	2	95.44 ± 0.24^{a}	-	-

A 50 cm filter height, a concentration 2 g/L and proportions of *S. cerevisiae* and *L. casei* 50:50, 66:33 and 77:23 of both microorganisms were selected in Test 4 to establish the effect of flow rate in mixtures of *S. cerevisiae* and *L. casei*. Table 5

shows the percentage of cell removal of mixtures of both microorganisms according to the flow rate. The results indicated that the CSTC filter of 50 cm can be used to treat contaminated water with a mix of *S. cerevisiae* and *L. casei* cells. A removal of 81-99.6% of cells was obtained in all cell mixtures percentages. This result shows that is possible to remove microbial cells of different sizes and dimensions from water and decontaminate it. The influence of the flow rate according with the mix cell concentration is significant ($p>0.05$) on removal effectiveness. However, in order to achieve a complete cell removal, the mix composition of *S. cerevisiae* and *L. casei* (66-33%, respectively) and flow rate of 0.7 L/day for the filtration system is necessary. Taking into account that most of the results lead in removal of microbial cell mixtures in the range of 90-100%, it can be concluded that this methodology has a great potential as cold pasteurization method for water.

Table 5. Effect of flow rate on the removal of a mixture of microorganisms composed different concentrations of *S. cerevisiae* and *L. casei* cells in water, using corn stalk tubular-cellulose (CSTC) filter with 50 cm of height. Mean ± Standard deviation (n=3).

Mix composition *S. cerevisiae / L. casei* (%)	**Flow rate (L/day)**	**Cell removal (%)**
50.00/ 50.00	0.5	98.03±0.84^{c}
	0.7	89.82±0.82^{b}
	1	86.17±0.68^{a}
66.66/ 33.33	0.5	94.17±0.80^{a}
	0.7	99.60±0.53^{b}
	1	93.49±0.69^{a}
76.92/ 23.08	0.5	86.36±0.63^{b}
	0.7	89.87±0.98^{c}
	1	81.45±0.49^{a}

3.4. Continuous cold pasteurization of water and orange juice using CSTC filter.

In order to review the operating conditions of the filtration system, a continuous cold pasteurization of water was carried out for 10 days. The water was contaminated with 2 g/L of *S. cerevisiae* and passed through the filtration system in a flow rate of 1.5 L/day. Those parameters were selected according to the previous results shown in Table 2 and 3. The Figure 4a shows the percentage of cell removal of *S. cerevisiae* during water continued pasteurization. The system worked effectively for 10 consecutive days and the yeast cells removal ranged from 94 to 96% (Figure 4a). This efficiency of the continuous cold pasteurization in water established that the previous operating conditions could be applied to orange juice treatment, as well.

The Figure 4b shows the percentage of cell removal of *S. cerevisiae* during orange juice continued pasteurization using CSTC material. The results achieved a maximum value of 97% at day 14, which is considered satisfactory. The cell yeast removal range was in agreement with previous studies conducted on apple juice (Gialleli et al., 2016) using tubular cellulose materials from wood. To maintain operational stability, two filter regenerations washing with hot water, were carried out on the 4th and 12th day of operation. The average water consumption and time needed for each regeneration was 3 ± 0.5 L, supplying each time the water continuously in the vertical filter for 60 ± 5 min.

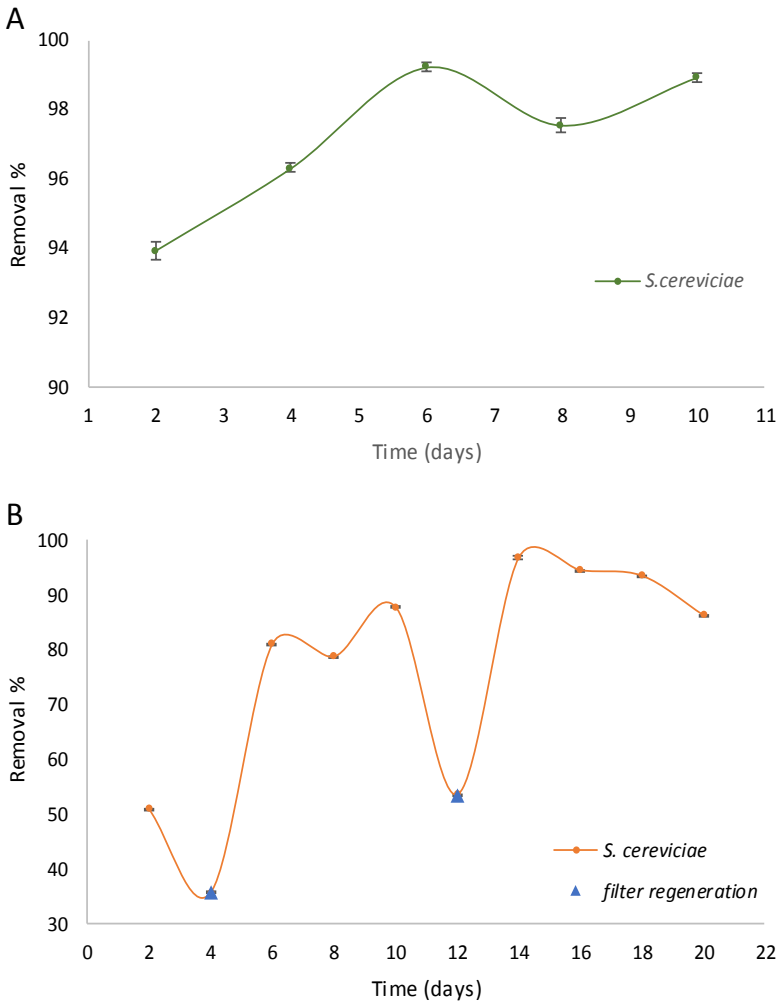

Figure 4. Percentage of cell removal of *S. cerevisiae* during the continuous treatment of water (A) and orange juice (B) using corn stalk tubular cellulose (CSTC) filter, with the following conditions: inlet concentration 2 g L^{-1}, flow rate of 1.5 L/day and 50 cm of filter height (means and standard deviations, n=3).

The results obtained in this first approach encourage the use of agricultural by-products as an excellent source of cellulose, to create alternative filtering materials. To achieve technological transfer, it is necessary to scale the proposed system to a pilot prototype for the citrus juice industry. Future studies should be carried out in

order to optimize this technology, taking into account the filtration parameters, testing in different microbial strains and food matrices.

3.5. Effect of the process on color

The proposed process is considered to be advantageous over the conventional thermal pasteurization, eliminating the risk of food quality deterioration caused by heating. At the same time, undesirable microorganisms can be effectively removed with low energy consumption and lower capital costs compared to expensive membrane filtration. However, the Figure 5 shows a loss of the color intensity of orange during the cold pasteurization process using CSTC. The color of orange juice treated presented some changes with regard to the untreated orange juice (0.9 ± 0.01) due to clarification process and retention of carotenoid contents. After regeneration of the filter the first measure of color index is even lower, which may be due to the residual water that is in the filter; however, in the subsequent measures the color intensity progressively increases reaching values similar to the untreated juice after 8 days of the regeneration. This cycle (decrease of color index and increase after the filter regeneration) is repeated throughout the filtration process, as it has been observed in other studies (Gialleli et al., 2014, 2016)

Fruit juices processed by non-thermal treatments present less color index than the pasteurized one (Cortes et al., 2007). This thermal treatment produces losses of carotenoid contents, especially of violaxanthin, antheraxanthin, and cisviolaxanthin, which means a perceptible color change (becoming lighter and more saturated) during traditional orange juice pasteurization (Lee & Coates, 2003). In the present study the color is not stable, which is an important disadvantage because the juice obtained by this method would not be homogeneous in this parameter.

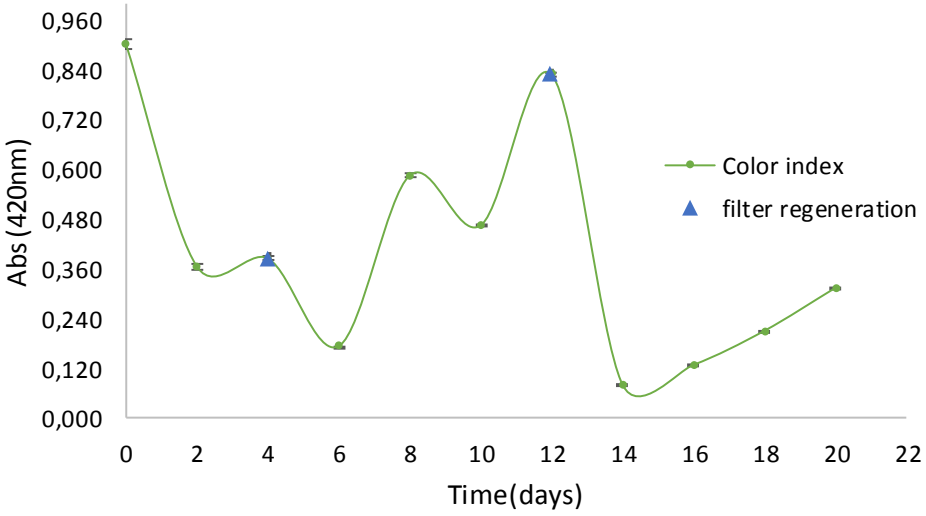

Figure 5. Color index of orange juice after cold pasteurization (means and standard deviations, n=3).

4. Conclusions

The CSTC was effective as filtering material for the removal of *S. cerevisiae* and *L. casei* from water. The flow rate played a crucial role on the microbial load removal efficiency. By increasing the height of the filter, the microbial removal was improved, especially in the case of CSTC. The microorganisms' removal capacity of CSTC material was 90-100% in the mixtures of *S. cerevisiae* and *L. casei.* The proposed system was also effective for the treatment of commercial orange juice contaminated with yeast cells in a continuous mode. The color of the juice was not homogeneous during continuous pasteurization. The results encourage the scale-up of the process, although some additional studies mainly focused on the effect of the process on the sensory and nutritional properties of orange juice (e.g. flavor, vitamin C, phenolic content fiber content) are needed. In general, CSTC constitute a low-cost porous material, light, abundant and of food grade purity with important potential for cell removal.

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5.2. Development of amino-functionalized membranes for removal of microorganism

Nataly Peña-Gómez[a], María Ruiz-Rico[a]*, Isabel Fernández-Segovia[a], José M. Barat[a]

[a]Grupo de Investigación e Innovación Alimentaria. Departamento de Tecnología de Alimentos, Universitat Politècnica de València. Camino de Vera s/n, 46022, Valencia, Spain

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Abstract

Treatments to ensure water supply of an acceptable hygienic-sanitary quality is of vast importance. Among unconventional treatments, membrane technologies have recently stood out. Immobilization of antimicrobial compounds onto membranes can prevent fouling and lead to self-cleaning matrices. In this study, cellulose membranes functionalized with amines were developed to assess their capability to remove microbial contamination. Water samples with several levels of *Escherichia coli* inoculum were filtered through membranes, and different trials were run to check the system's effectiveness. The amino-functionalized membranes were able to filter water samples in a few seconds, and partially or completely remove the inoculated microorganism depending on the inoculum level. The amine-functionalized membranes displayed significant retention capacity in samples with high bacterial concentrations and were able to decontaminate water with low microbial load. Membranes can be reused with no apparent loss of efficiency. Hence, this study demonstrates the high potential of amine-functionalized membranes in drinking water treatments.

Industrial relevance

Filtration represents an important non-thermal process used for clarification, concentration and microbial stabilization of liquid fluids. However, membrane fouling and related cleaning requirements are critical factors determining the extensive application of this technology. This work represents an important starting point to the development of new antimicrobial surfaces due to the demonstrated advantages associated to the covalent immobilization for the development of novel filtration treatment methodologies.

Keywords: *Escherichia coli*; filtration; immobilization; cellulose; polyamines; disinfection.

1. Introduction

Water is essential for all living beings, which makes ensuring its safety in the moment when consumed a priority. Drinking water is defined as the water used for domestic purposes, such as drinking, cooking and personal hygiene, and is considered safe if it meets certain microbiological and chemical standards (World Health Organization, 2017). Unfortunately, the world's entire population cannot access necessary water supplies, and developing countries are particularly suffering serious water shortage consequences. Every year, millions of people suffer from different illnesses, such as cholera, diarrhea, dysentery, typhoid fever, etc., caused by drinking contaminated water. In fact it has been calculated that more than 502,000 people die per year due to diarrhea caused by drinking unsanitary water according to the WHO (World Health Organization, 2011).

Drinking water can be contaminated by chemical compounds, physical elements and biological species, such as viruses and bacteria, which pose a direct human health risk (Kroll et al., 2012). Pathogens derived from faeces are the main concerns for establishing health-based objectives for microbial safety. Thus, the WHO (2017) has set the absence of fecal coliforms, specifically *Escherichia coli*, *Enterococcus* and *Clostridium perfringens*; per 100 mL of water as a microbiological limit.

The growing demand of drinking water is today a worldwide challenge (Tiwari, Tiwari, Behari, & Sen, 2008). Therefore, running appropriate treatments to ensure that water supplies offer an acceptable hygienic-sanitary quality is of much importance. Approaches for ensuring microbial safety of drinking water are based on methods to prevent the contamination of drinking water or to reduce contamination to levels not adverse to health. The use of chlorine for water disinfection has been considered one of the best achievements of the 20th century in the public health field (Center for Disease Control and Prevention, 1999). However, toxic or carcinogenic disinfection by-products (DBPs) are formed from using chemical disinfectants, such as chlorine, chloramine and ozone (Richardson, Plewa, Wagner, Schoeny, & DeMarini, 2007). The specifically use of chlorine may

lead toxic compounds to form, such as trihalomethanes or chlorinated phenols, among others. Furthermore, the resistance of some pathogens to traditional chemical disinfectants renders it necessary to use extremely high doses, which means high DBPs levels in drinking water (Li et al., 2008). Hence, the pressing need to review conventional disinfection methods and to consider innovative approaches to both improve the disinfection reliability and avoid DBPs formation.

Different water treatment methodologies have been proposed to ensure water quality without affecting consumers health to prevent water shortage problems (Li et al., 2008). Membrane technologies stand out among the alternative water treatment systems available to treat wastewater, drinking water and water production (Salehi, 2014). Membranes allow the bacteria cells to be retained and provide purified water free of bacterial contaminants and DBPs (Kroll et al., 2012). This technology specially highlights in household water treatment (HWT) or point-of-use water technologies to treat collected water or contaminated piped water in developing countries. According to the guidelines for drinking-water quality stated by the WHO, the bacterial reduction achieved by membrane filtration in household water treatment technologies falls within the range of 1-2 or 2-4 $\log_{10}$ reduction value (LRV), for fiber and fabric filters and microfiltration, respectively (World Health Organization, 2011). However, membrane fouling is the most critical problem in membrane technology given the presence of biomolecules or microorganisms in water samples that hinder the water flux.

Immobilization of bioactive compounds onto membrane surfaces can prevent fouling and lead to self-cleaning matrices (Gialleli, Bekatorou, Kanellaki, Nigam, & Koutinas, 2016; Kroll et al., 2012). The use of functionalized surfaces by anchoring active inorganic compounds, e.g. silver (Dankovich & Gray, 2011; Gopiraman et al., 2016; Lala et al., 2007; Oyanedel-Craver & Smith, 2008; Sinclair, Zieba, Irusta, Sebastián, & Arruebo, 2014; Tankhiwale & Bajpai, 2009) or graphene (Hu et al., 2010), and active organic compounds, e.g. essentials oils (Royo, Fernández-Pan, & Maté, 2010), peptides (Nakamura et al., 2011), biocides (Mansur-Azzam, Woo, Eisenberg, & van de Ven, 2013) and enzymes (Kroll et al., 2012) has been recently

proposed. This methodology provides simple, portable and cheap disinfection systems that do not require special equipment and efficiently reduce microbial density (Dankovich & Gray, 2011; Mansur-Azzam et al., 2013). There is no doubt that antimicrobial membranes are better than other traditional techniques for water treatment, but more studies still need to be done to assess the environmental impact of immobilized compounds (Tiwari et al., 2008). An alternative to the aforementioned systems could be the immobilization onto membrane surfaces of organic compounds, such as amines, which form part of some of the most important biological compounds that act as bioregulators, neurotransmitters, in defensive mechanisms and many other functions in living beings (Igarashi & Kashiwagi, 2010). Some polyamines, such as quaternary amines, have been widely reported as antimicrobial agents. Quaternary ammonium compounds with N-alkyl chains present antimicrobial activity given the association between positively charged quaternary nitrogen and negatively charged head groups of acidic phospholipids in bacterial membranes, which disrupts membrane integrity (Buffet-Bataillon, Tattevin, Bonnaure-Mallet, & Jolivet-Gougeon, 2012). Quaternary amines have been successfully anchored to membranes to create antimicrobial supports (Andresen et al., 2007; Kim, Nam, Park, & Park, 2007; Roy, Knapp, Guthrie, & Perrier, 2008). However, the application of such amines has been related to some toxicological issues and microbial resistance (Aase, Sundheim, Langsrud, & Rørvik, 2000; Thorsteinsson et al., 2003). Otherwise, primary and secondary amines are not considered effective antimicrobial agents. Bartels et al. (2016) developed amino-silanized yttria stabilized zirconia capillary membranes for controlled virus retention based on the positive surface charge of the amino groups immobilized on the membranes for the adsorption of the negatively charged viruses. Following a similar approach in this study, we evaluated the development of cellulose membranes functionalized with a primary amine and assessed their capability to remove *Escherichia coli* in water samples.

2. Materials and Methods

2.1. Chemicals

Standard paper grade cellulose paper (75 g/m^2) (product number RM13054252) was purchased from Labbox (Barcelona, Spain). *N*-(3-trimethoxysilylpropyl)diethylenetriamine (N3) (product number 413348) and dimethyl sulfoxide (DMSO) (product number 1029522511) were provided by Sigma-Aldrich (Madrid, Spain).

2.2. Preparing functionalized membranes

The surface of cellulose membranes was covalently functionalized with polyamines to create antimicrobial membranes for water sterilization. For that purpose, polyamine *N*-(3-trimethoxysilylpropyl)diethylenetriamine was covalently anchored to paper membranes. In a typical experiment, a solution of N3 (50% v/v) in dimethyl sulfoxide was prepared (Akhlaghi et al., 2015). Then, 1.5 mL of the mixture were added drop by drop to the paper membranes (ø 4.5 cm). Once membranes were well impregnated, they were dried at room temperature for 2 h. Finally, membranes were washed with distilled water (100 mL) to remove excess polyamines and were dried at 40°C for 24 h.

2.3. Membrane characterization

Paper membranes were characterized following standard instrumental techniques. A morphological analysis of the cellulose microstructure was performed by Field Emission Scanning Electron Microscopy (FESEM) observations. FESEM images were acquired with a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. The space between fibers (pore size) was estimated by averaging the measured size values of 50 replicates. Confirmation of membrane functionalization was determined by Fourier transform infrared spectroscopy (FT-IR), thermogravimetric analyses (TGA)

and orange II assay. IR spectrums of membranes were obtained with a Bruker Infrared Spectroscopy Tensor 27 instrument (Massachusetts, US) between 4000 and 400 cm^{-1} in the transmittance mode. TGA determinations were made on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc., Schwarzenbach, Switzerland), using a heating program that consisted in a heating ramp of 10 °C/min from room temperature to 600ºC in a nitrogen atmosphere (50 mL/min). The quantification of the amount of accessible amino groups was determined by the orange II analysis based on the principle that one acid orange dye molecule binds to one amine group (Kroll et al., 2007). Amine-functionalized membranes were incubated in 1.75 mL solution of 0.5 mM acid orange II sodium salt in HCl (pH 3) for 24 h. After incubation, membranes were washed with abundant HCl solution (pH 3) to remove unbound dye molecules. The desorption of the bound orange II molecules was performed by pH-shift adding 2 mL NaOH solution (pH 12) followed by incubation for 15 min under shaking. The supernatants were spectrophotometrically analyzed at 483 nm using NaOH solution (pH 12) as blank sample and comparing the absorbance with that of NaOH solution with acid orange II at various concentrations. The potential leaching of the immobilized aminosilanes was evaluated after washing of the membranes with 1 L of sterile water and then quantification of the accessible amino groups attached to the washed membranes by the orange II assay.

2.4. Microbiological assays

The bacterial strain used in the microbiological studies was a non-pathogenic strain of *Escherichia coli* K12 (CECT 433), obtained from the Colección Española de Cultivos Tipo (CECT; Valencia, Spain). This strain was chosen for its role as a fecal contamination indicator in water according to the WHO (2017). Plate Count Agar (PCA) (product number 01-161-500) and Tryptone Soy Broth (TSB) (product number 02-200-500) were used to grow the microorganism. Peptone water (product number 02-568-500) and sterile distilled water were used to prepare decimal dilutions of the inoculum. Selective media Tryptone Bile X-Glucuronide (TBX) agar

(product number 01-619-500) was used to plate the microorganism after treatment. All the media were provided by Scharlab (Barcelona, Spain).

2.4.1. Inoculum and inoculated water preparation

The bacterial strain was reconstituted following the CECT instructions. Bacterial stock was stored at 4 ºC in PCA before use. The cells from an *E. coli* colony grown on PCA were transferred to 10 mL of TSB and were incubated at 37ºC for 24 h to obtain an inoculum with a density of approximately 1 x 10^9 cells/mL of broth. Different inoculum densities were tested from 10 to 10^8 CFU/mL of water. For this purpose, decimal dilutions were prepared in peptone water and subsequently, according to the studied inoculation density, the last dilution was prepared in sterile distilled water to obtain test tubes with 10 mL of contaminated water.

2.4.2. Water filtration assays

The filtration procedure was carried out using a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany) linked to a pump with a 6 L/min speed pressure/suction and connected to a conic polystyrene tube to collect the filtered sample.

Four different filtration tests were performed as follows:

Test 1: Study the retention capacity of the amine-functionalized membranes against five inoculum densities (10^4, 10^5, 10^6, 10^7 and 10^8 CFU/mL) by assuming the worst-case scenario to test the filtration efficacy of the developed membranes. In a typical experiment, 10 mL of inoculated distilled water (pH 6.10 ± 0.09) were filtered through a functionalized membrane and the permeate (>9.9 mL) was recovered in a conic polystyrene tube.

Test 2: Assess the efficacy of membranes by increasing the number of filtrates or the number of membranes. To this end, a distilled water sample with a microbial density of 10^4 or 10^6 CFU/mL was filtered 3 times with the same membrane or was

filtered with three different membranes or three membranes together following the above-explained procedure.

Test 3: Study the reuse capability of the developed membranes. In this test, five different distilled water samples, with a microbial density of 10^4 or 10^6 CFU/mL, were filtered through the same membrane.

Test 4: The retention capacity of the amine-functionalized membranes was tested in distilled water samples using low microbial concentration having established the antimicrobial activity of the developed membranes against cultures in the stationary phase with a high microbial load. Regulatory agencies define a moderate risk of the presence of >10 *E. coli* per 100 mL (Bain et al., 2014) and a very high risk exists when the number of coliforms per 100 mL is above 10^3 CFU (World Health Organization, 2011). Therefore, for this experiment 10 mL of water were inoculated with a bacterial density of 10 or 10^2 CFU/100 mL. Contaminated water was filtered through the amine-functionalized membrane and a sterilizing membrane filter (0.45 µm pore size) (EZ-Pak, Merck Millipore, Molsheim, France) that retained the microorganisms on its surface after the filtration procedure. The same tests were carried out with the non-functionalized membranes used as a positive control. All the tests were performed in triplicate.

The antimicrobial activity of the filtration system was quantified by preparing serial dilutions of filtrates and plating them on selective media or by transferring the sterilizing filtration membranes to the culture media. Plates were incubated at 37 ºC for 24 h and viable cell numbers were determined as colony-forming units per mL (CFU/mL). These values were logarithmically transformed and expressed as log CFU/mL. Control positive values (water samples filtered through a non-functionalized cellulose membrane) were used to quantify the microbial count in the absence of treatment and to then calculate the percentage of bacterial reduction (in logarithmic basis) and the log reduction value (LRV).

2.5. Statistical analysis

Data were statistically analyzed with Statgraphics Centurion XVI (Statpoint. Technologies, Inc., Warrenton, VA, USA). The influence of different variables on bacterial viability was analyzed by analyses of variance (one-way and multifactor ANOVA). The LSD (least significant difference) procedure was used to test the differences between averages at the 5% significance level.

3. Results and Discussion

3.1. Membrane characterization

The non-functionalized and amine-functionalized paper membranes were characterized using FESEM, Fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analyses (TGA), as mentioned above. Figure 1 shows the microstructure of the non-functionalized (a, b) and the amine-functionalized (c, d) paper membranes obtained by FESEM.

As observed in Figure 1a and c, the untreated paper membrane is formed by cellulose fibers that are more compact than the functionalized paper membrane. The amine-functionalized paper appears to have more separated cellulose fibers than non-modified membranes, which may be result from functionalization and the washing treatment. However, these differences are not observed in Fig. 1b and d, which show the fibrillary structure in more detail. According to FESEM analysis, control membranes present a mean pore size of ca. 38.9 ± 14.3 µm, whereas amine-functionalized membranes showed a pore size of ca. 41.4 ±.17.2 µm. In either case, the cellulose paper microstructure allowed microorganisms to pass through spaces of up to 10 µm between fibers at a reasonably fast gravity flow without resorting to pressure or suction (Dankovich & Gray, 2011), and also favored direct contact between bacteria and the immobilized polyamines. Fourier transform infrared spectroscopy (FT-IR) was carried out to confirm the attachment of polyamines to the cellulose paper surface qualitatively.

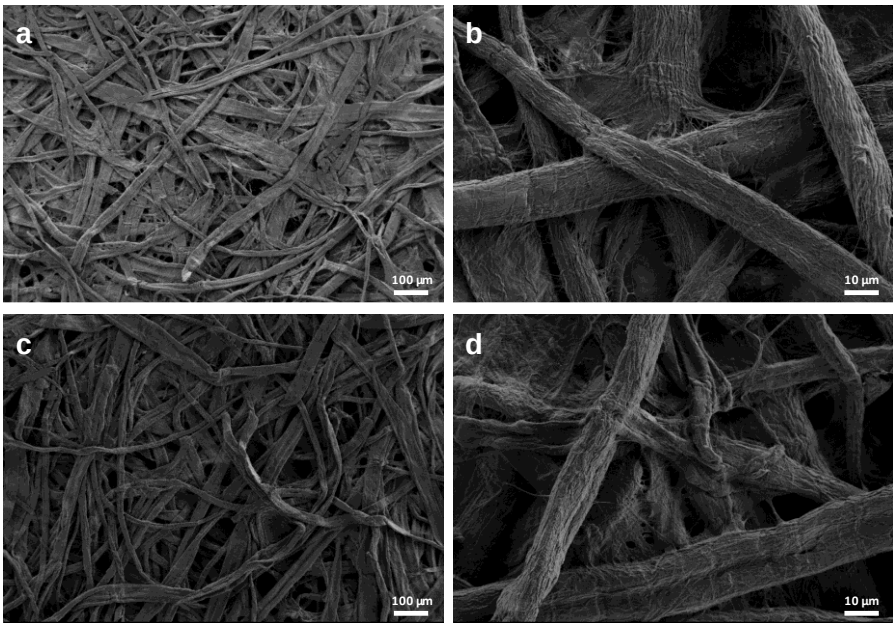

Figure 1. FESEM images of the microstructure of the non-functionalized cellulose membrane (a, b) and the cellulose membrane functionalized with *N*-(3-trimethoxysilylpropyl) diethylenetriamine (c, d).

Figure 2 shows the FT-IR spectra of the non-functionalized cellulose membrane and the amine-functionalized cellulose membrane. As we can see, the control cellulose membrane (upper line) presents a large band within 2000 to 2500 cm^{-1} wavelength range, which corresponds to the CH_2-OH bonds of the cellulose molecule. Low-intensity peaks appear at a wavelength above 3500 cm^{-1} due to the presence of absorbed water being weakly bound that falls within 1600 to 1700 cm^{-1} range because of adsorbed water (Schwanninger, Rodrigues, Pereira, & Hinterstoisser, 2004). In contrast, the amine-functionalized cellulose membrane presents other representative bands. In this case, the band that corresponds to the CH_2-OH bonds and the peaks that corresponds to the presence of water are preserved, but new bands emerge, which confirms the immobilization of amines on the cellulose material surface. The presence of amines is observed on the peaks

that correspond to N-H and NH_2 bonds, which fall within the 3000 to 3500 cm^{-1} range and the 1500 to1600 cm^{-1} range, respectively. The attachment of the trialkosysilane to the cellulose membrane is shown in the bands at 3700 cm^{-1} and 1200 cm^{-1}, which respectively correspond to the Si-OH and Si-CH_2 bonds, as reported in other studies (Hiyoshi, Yogo, & Yashima, 2005; Pacheco, Johnson, & Koros, 2012; Tumuluri, Isenberg, Tan, & Chuang, 2014).

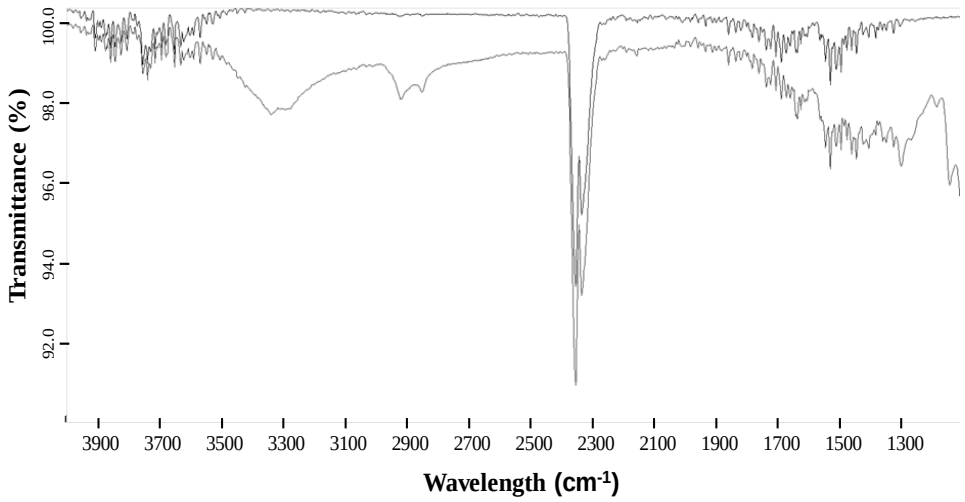

Figure 2. FT-IR spectra of the control cellulose membrane (upper line) and the cellulose membrane functionalized with *N*-(3-trimethoxysilylpropyl) diethylenetriamine (lower line).

Thermogravimetric analyses were carried out to confirm the immobilization of amines to the cellulose support. Table 1 shows the percentage of weight loss according to the different temperature ranges of the non-functionalized cellulose membrane and the amine-functionalized cellulose material. The non-modified cellulose membrane showed almost total degradation within the range of tested temperatures. Initially slight degradation was observed from room temperature to 100ºC, which corresponded to residual water. Major weight loss took place between 100ºC and 600ºC, which corresponded to 95.74% loss of organic matter,

which means that the sample had almost entirely burned. This behavior is similar to that observed in previous studies (Yang, Yan, Chen, Ho, & Zheng C, 2007; Zhu, Sui, Wang, Sun, & Sun, 2004). In contrast, the N3-functionalized cellulose thermogram showed a different behavior and confirmed correct membrane functionalization. The initial mass loss (25-100ºC), which corresponded to water, represented a greater loss than for the control sample because of the functionalization and washing processes.

Table 1. Percentage of weight loss of the non-functionalized and amine-functionalized cellulose membranes according to different temperature ranges.

Membrane type	25 – 100 ºC	100-300 ºC	300-600 ºC
Non-functionalized cellulose	1.75%	46.69%	47.30%
Amine-functionalized cellulose	4.97%	40.69%	24.61%

The second stage was similar to the non-functionalized membrane, but the percentage of weight loss was significantly lower in the third step. Total loss of matter was 70.27%, which is a much lower value than that of the control sample due to the presence of the trialkoxysilane group of immobilized amines, which degraded at higher temperatures. These results are in accordance with previous studies where the pyrolysis of amine ligands was complete by 700ºC (Zelenak, Halamova, Gaberova, Bloch, & Llewellyn, 2008), and the thermal decomposition residue of cellulose membranes after 600ºC was higher after the silylation with 3-aminopropyl trimethoxysilane by the increase of silicon associated to the alkoxysilane ligands (Saini, Belgacem, Salon, & Bras, 2016).

After confirmation of the attachment by qualitative methods, the accessible NH_2-groups/nm^2 on the amine-functionalized membranes were quantified by the acid orange II assay. The analysis of different modified membranes showed a mean amount of 10.11 ± 0.98 NH_2-groups/nm^2. The functionalization with N3 as an aminosilane with three amino groups per silane molecule resulted in a high amount of accessible NH_2-groups on the membranes' surface that can adsorb the microbial

cells (Bartels et al., 2016). In addition, the potential leaching of the immobilized aminosilanes was evaluated by orange II assay after washing the membranes with 1 L of sterile water.

The determination of the accessible amino groups attached on the membranes resulted in a mean amount of 10.86 ± 1.71 NH_2-groups/nm^2. Therefore, no wash-out effect after filtering high volume of water was observed, which confirms the covalent immobilization of the aminosilanes onto the membrane surface.

3.2. Bacterial retention assessment of the amine-functionalized membranes

The adsorption capacity of the amine-functionalized cellulose membranes was assessed against a high bacteria load (between 10^4 and 10^8 CFU/mL) by assuming the worst-case scenario to test the filtration efficacy of the developed membranes, as reported in other studies (Sinclair et al., 2014). Having established the inhibitory capability of membranes, the antimicrobial effect of the amine-functionalized cellulose membranes was evaluated in the water samples by simulating low microbial contamination with a bacterial density of 10 or 10^2 CFU/100 mL (Bain et al., 2014), as mentioned above.

The membrane flux of the non-functionalized and amine-functionalized cellulose membranes was in the same range (permeate volume >9.9 mL and filtration in less than 10 s), given the preservation of the membrane pore size after the immobilization process (Figure 1).

3.2.1. *Retention activity of the amine-functionalized cellulose membranes according to inoculum density*

Figure 3 shows the percentage of microbial reduction of the water samples inoculated with *E. coli*, with an initial inoculum density that range from 10^4 to 10^8 CFU/mL after filtration with an amine-functionalized cellulose membrane. The filtration of inoculated water through the non-functionalized membranes did not

reduce the microbial load (data not shown). The developed membranes displayed a significantly different antimicrobial effect against *E. coli* depending on the inoculum density level ($p<0.05$), which was the biggest difference for the lowest bacterial load (10^4 CFU/mL). A reduction of 52±4% in logarithmic basis (equivalent to reduction of 99.96% of CFU) in the initial inoculum was achieved for inoculum density 10^7 CFU/mL.

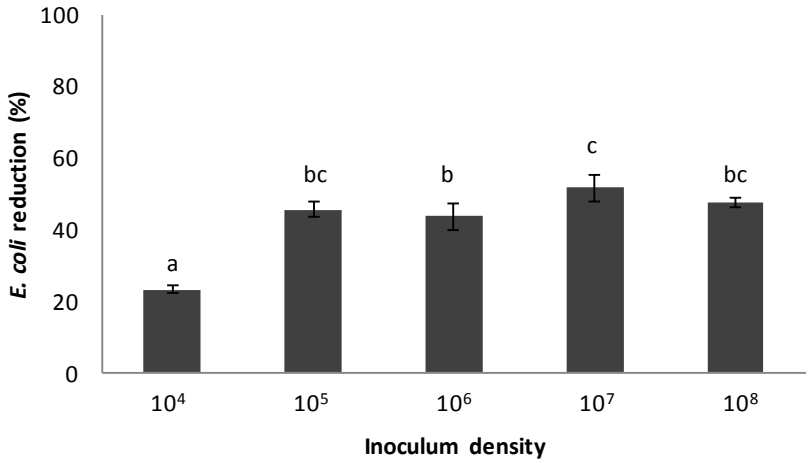

Figure 3. Percentage of reduced *E. coli* (after filtration with membranes with *N*-(3-trimethoxysilylpropyl) diethylenetriamine according to inoculum density. Different letters on the bars indicate significant differences ($p<0.05$) (means and standard deviations, n=3).

The maximum inhibition level was obtained for the highest inoculum density, obtaining a reduction of 3.74 ± 0.11 LRV. Although the maximum inhibition percentage did not exceed half of the initial microbial population, it is noteworthy that an inoculum was used in the stationary phase with a high microbial load, which did not simulate a real scenario. Furthermore, the inhibitory capability of the amine-functionalized membranes was remarkable if we take into account that the inoculated water filtration required only a few seconds.

The results obtained after single filtration of water contaminated with high microbial load through the amine-functionalized membranes meet the requirements stated by WHO for the point-of-use water technologies based on membrane, porous ceramic or composite filters. According to the guidelines for drinking water quality, filtration through fiber and fabric filters should reduce 1-2 LRV (World Health Organization, 2011), and these range was achieved by the amine-functionalized membranes for most of the inoculum densities (from 0.91 ± 0.05 to 3.74 ± 0.11 LRV, according to increasing inoculum level).

The bacterial retention capacity of the amine-functionalized membranes is related to the positively charged surface of the fibers due to the immobilization process. Despite zeta potential has not been analyzed in this study, previous authors have stated the conversion of the zeta-potential of the membrane surface from negative (non-functionalized) to positive values after amine-functionalization (Bartels et al., 2016). Due to the positive membrane surface charge of the amine-functionalized membranes, with high content of accessible amino moieties, the negatively charged bacteria (Habimana, Semião, & Casey, 2014) were adsorbed onto the membranes' surface through the filtration process.

The retention capability of a surface functionalized with N3 has been reported by immobilization of this polyamine on yttria-stabilized zirconia capillary membranes for controlled virus retention (Bartels et al., 2016). Virus retention tests showed the substantial virus retention efficiency of the aminosilanized membranes after filtration of a viral solution through the capillary membranes. Amino-functionalized ceramic membranes displayed LRV levels of 9, being the result of the positive charge of the membranes that able to adsorb the virus because of the high content of accessible amino groups.

Other examples of the immobilization of bioactive compounds with antimicrobial activity onto cellulosic surfaces have been reported, despite they were not used as filtering elements. Saini et al. (2016) described the functionalization of cellulose nanofibers with other silane like 3-aminopropyl trimethoxysilane. The antimicrobial activity of the non-leaching antimicrobial

surface was evaluated against different bacteria by zone inhibition test and quantitative antibacterial method, obtaining a reduction in bacterial concentration of 3.8 log for *E. coli* after 24 h of incubation of the inoculated films. A quaternary ammonium compound was grafted onto the surface of microfibrillated cellulose to develop non-leaching antimicrobial films.

The antibacterial activity of the films was assessed by the zone inhibition test obtaining significant reduction in viable microorganisms (Andresen et al., 2007). Enzymes were anchored to modified cotton fabrics and their antimicrobial activity against bacteria and fungi was evaluated being able to inhibit the microorganisms even after consecutive wash cycles (Ibrahim, Gouda, El-shafei, & Abdel-Fatah, 2007).

3.2.2. *Retention activity of the amine-functionalized cellulose membranes according to the number of filtrates or the number of membranes*

After establishing the adsorption potential of the amine-functionalized membranes, the retention efficacy of membranes was evaluated after successive filtrations. To this end, one water sample with an inoculum density of 10^4 or 10^6 CFU/mL was filtered 3 times with the same amine-functionalized membrane, and one sample was filtered 3 times with three amine-functionalized membranes or was filtered once using three membranes together.

Figure 4 presents the reduction percentage for *E. coli* after successive filtrations. The samples subjected to three filtrates obtained an inhibition average value of 31 ± 8 and 38 ± 3% for the 10^4 and 10^6 CFU/mL inoculum densities, respectively. The results were significantly different after three filtrations compared to the single filtration (Figure 3). The samples filtered 3 times with three amine-functionalized membranes presented a similar inhibition percentage to the three filtrates, for the 10^4 CFU/mL inoculum density, but the inhibitory effect was higher for that of 10^6 CFU/mL with a bacterial reduction of 51 ± 6%. The water samples subjected to filtration through three membranes together presented

significant higher reduction (41 ± 7 and 46 ± 0% for the low and high inoculum density, respectively). These results are in accordance with the above-described test (Figure 3), where the higher the bacterial concentration, the stronger the retention effect of the developed membranes.

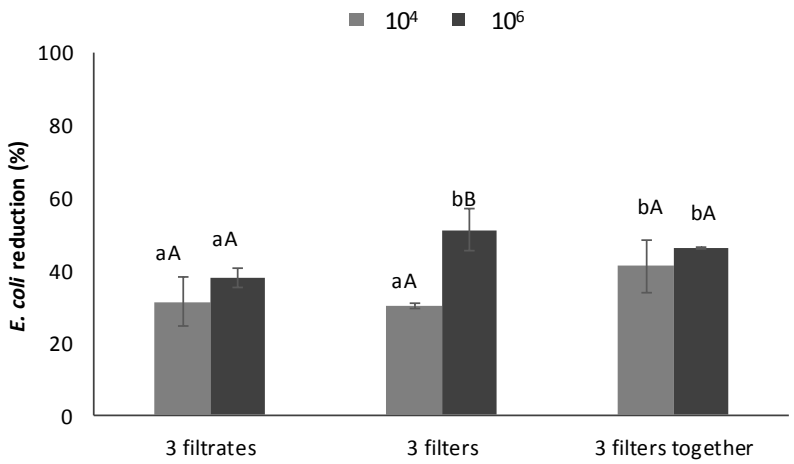

Figure 4. Percentage of reduced *E. coli* after filtration of one sample with one amine-functionalized membrane 3 times (3 filtrates) or after filtration of one sample with three amine-functionalized membranes (3 filters) or three membranes together (3 filters together). Different letters on the bars indicate significant differences ($p<0.05$) from the levels of inoculum densities (small letters) and the assay type (capital letters) (means and standard deviations, n=3).

Similar to the results of Test 1, a reduction above 1 LRV was achieved after filtering the inoculated water (10^4 CFU/mL) through different membranes or after successive filtrations. For water inoculated with the high bacterial load (10^6 CFU/mL) the retention capacity resulted in 2-3 LRV, higher reduction than the values established in the guidelines given by the WHO for filtration technologies based on fiber and fabric filters (World Health Organization, 2011).

In spite of the slightly enhanced adsorption activity obtained in this test, it would be necessary to carry out studies with a growing number of filtrates to

establish the increase in antimicrobial activity after repeated filtrations (up to 50 times) as described in previous works (Sinclair et al., 2014).

3.2.3. *Study of the reuse capability of the amine-functionalized membranes*

The feature studied in this test was the reuse capability of the amine-functionalized membranes after filtering multiple samples. In a typical experiment, five water samples with a bacterial load of 10^4 or 10^6 CFU/mL were filtered through the same membrane, as explained in Section 2.4.2. Figure 5 shows the percentage of *E. coli* reduction after filtering the different samples. The retention of the bacteria population was boosted with a bigger number of filtrates, but samples did not significantly differ according to inoculum density or number of sample.

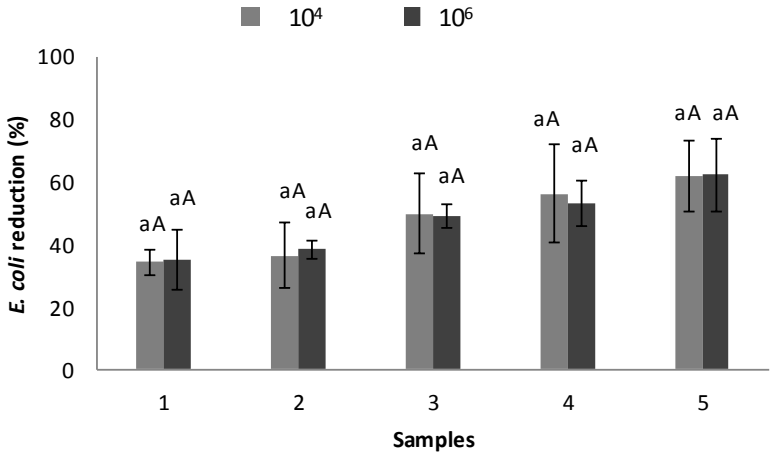

Figure 5. Percentage of reduced *E. coli* after filtration of five different samples with one amine-functionalized membrane. Different letters on the bars indicate significant differences ($p<0.05$) from the levels of inoculum densities (small letters) and the sample (capital letters) (means and standard deviations, n=3).

A certain growing trend in the inhibitory effect was observed with a larger number of filtered samples, which could be caused by a rise in the hydration grade

of cellulose fibers after successive filtrates. Hydration could generate an increased fiber surface by reducing pore spacing, which could enhance the contact between samples and the polyamines attached to the cellulose membrane surface. The microstructure of cellulose fibers after hydration was studied by microscopic analysis, but this hypothesis could not be supported by the obtained results because the fibers of the membranes did not present significant differences in morphology and pore size (data not shown).

The reuse capability results indicated that the inhibitory effect was maintained while filtering the different samples because of the immobilization of the active molecule (polyamines) through a covalent bond. The results are in accordance with the quantification of the accessible amino groups results, which showed the preservation of accessible amino groups onto the membranes' surface after washing with high volume of water. This approach for obtaining membranes with antimicrobial properties, in comparison with non-covalent binding methods, present the main advantage of the prevention of the release of the immobilized compounds, which can affect the properties of the treated matrix (Barbiroli et al., 2012).

3.2.4. *Study of the retention capability of the amine-functionalized membranes on water samples with low microbial load*

Finally, the adsorption activity of the amine-functionalized membranes was evaluated in the water samples by simulating the minimum microbial load that implies a risk for human health (Bain et al., 2014). To do this, samples were filtered through one amine-functionalized membrane 1 and 3 times, and were successively filtered through three membranes or were filtered once using three membranes together. A sterilizing membrane filter (mixed cellulose ester, 47 mm, 0.45 µm) was included at the bottom of the filtration system for microbial enumeration purposes, and also because its pore size allowed the microorganisms on the membrane surface to be retained after sample filtration.

Table 2 shows the microbial growth of *E. coli* in the water samples (inoculated with 10 and 10^2 CFU/100 mL) after filtering with the amine-functionalized membranes. Filtration through one membrane reduced both inoculum densities by around 40%. Filtering the water samples 3 times through one membrane enhanced the membranes' retention effect. The most remarkable results were obtained by successively filtering samples through three membranes or filtering them once using three membranes as an ensemble on the filtration device. The use of multiple membranes greatly removed the microorganism (a reduction around of 80-100%) after the filtration procedure. These results confirmed again the potential of the amine-functionalized membranes as filtering elements due to their retention capacity (1-2 LRV) according to the guidelines for drinking water quality.

Table 2. Microbial growth of *E. coli* (log CFU/100 mL) in contaminated water after filtration with the amine-functionalized membranes (means and standard deviations, n=3).

	Inoculum concentration (log CFU/100 mL)	
Treatment	**10**	**10^2**
Control	0.82 ± 0.10^{aB}	1.72 ± 0.04^{aA}
1 membrane	0.48 ± 0.00^{bB}	1.11 ± 0.04^{bA}
3 filtrates	0.23 ± 0.40^{cA}	0.30 ± 0.00^{dA}
3 membranes	0.00 ± 0.00^{dB}	0.78 ± 0.15^{cA}
3 membranes together	0.00 ± 0.00^{dA}	0.15 ± 0.21^{eA}

Different superscripts denote differences ($p<0.01$) among count values from treatment conditions levels (small letters) and between inoculum densities (capital letters)

The adsorption capability of the amine-functionalized membranes is based on the electrostatic attraction that takes place between the activated positively charged membrane surface and the negatively charged microorganism. This binding, between the bacteria envelope and the accessible amino groups attached onto the surface of the cellulose membranes, might allow the attack of the cell wall

with a local concentration of amines so high that it could easily disrupt the cell membrane and to induce the cell death (Huang, Wang, & Yan, 2010; Zhan et al., 2014).

In this work, the microbial retention capability of the developed membranes was performed with distilled water, where the only contaminant was the target bacterium. For drinking water treatment, a potentially significant disadvantage is membrane fouling by organic matter present in water that can be negatively charged and therefore able to adsorb to the positively charged membrane surface. Therefore, further studies with real water samples are necessary to validate the approach of bacterial removal.

4. Conclusions

In this work, a proof of concept of new antimicrobial membranes for water treatment was developed. Antimicrobial membranes were prepared through the covalent immobilization of polyamines on the surface of commercial cellulose paper. The amine-functionalized membranes showed excellent retention capacity in water samples with different inoculum densities for the Gram-negative microorganism *E. coli*. The results also demonstrated that the developed membranes can be used with either different filtrates or several membranes as an ensemble, and can be reused with no apparent loss of efficiency. The water contaminated with 10 CFU/100 mL of *E. coli* was completely sterilized after performing high-speed filtration with the functionalized cellulose membranes. Therefore, the present study demonstrates the very high potential of the developed membranes in drinking water treatment thanks to several advantages, such as speed, easy handling, low cost and potential *in situ* use.

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6. CHAPTER 2. DEVELOPMENT OF FUNCTIONALIZED SILICA SUPPORTS

6.1. Novel antimicrobial filtering materials based on carvacrol, eugenol, thymol and vanillin immobilized on silica microparticles for water treatment

Nataly Peña-Gómez[a], María Ruiz-Rico[a]*, Édgar Pérez-Esteve[a], Isabel Fernández-Segovia[a], José M. Barat[a]

[a]Grupo de Investigación e Innovación Alimentaria. Departamento de Tecnología de Alimentos, Universitat Politècnica de València. Camino de Vera s/n, 46022, Valencia, Spain

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Abstract

The objective of this work was to develop filtering materials based on different-sized silica particles functionalized with carvacrol (0.01 g/g SiO_2), eugenol (0.04 g/g SiO_2), thymol (0.01 g/g SiO_2) and vanillin (0.12 g/g SiO_2). The removal capability of the functionalized supports was evaluated using distilled water inoculated with *Escherichia coli* (10^4 CFU/mL). Water samples (100 mL) were filtered through layers of supports of different thickness (0.5, 1 or 1.5 cm thick), according to different filtration tests. The results showed the supports' efficacy, because the microorganism was totally eliminated after filtration, requiring the passage of the sample through the filter only a few seconds in some cases. Removal of *E. coli* was due to a combination of physical adsorption and inactivation on contact with the immobilized molecules. Functionalized supports' efficacy remained after filtering multiple samples and/or pre-conditioning (washing with 1-3 L of sterile water), which suggests the maintenance of the molecules attached to silica microparticles' surface. No wash-out effect was determined after filtration with eugenol, thymol and vanillin functionalized supports, which demonstrated the covalent immobilization of antimicrobial compounds.

Industrial relevance

Ensuring appropriate water decontamination with no potential health risks associated with conventional chemical disinfectants and meeting the growing point-of-use water treatment demand require emerging technologies for the microbial decontamination of drinking water. The developed filtering materials showed good bacterial removal capacity with log reduction values of 10^4 CFU/mL, which are adequate for household water treatment technologies. As proof-of-concept, this study demonstrated the high potential of the developed functionalized silica supports to remove bacteria such as E. coli in water treatment.

Keywords: drinking water; bioactive compounds; *Escherichia coli*; functionalized silica particles; covalent immobilization.

1. Introduction

Access to safe drinking water is essential to prevent the health risks associated with intake of microbiologically contaminated water. The absence of fecal coliforms per 100 mL of water is established as a microbiological limit for drinking water (World Health Organization, 2017) given the correlation between the presence of both fecal contamination and disease-causing microorganisms (Tallon, Magajna, Lofranco, & Leung, 2005).

For more than one century, sand filtration and chlorination have ended most waterborne epidemics in developed countries (Li et al., 2008). Despite their effectiveness, harmful disinfection byproducts are formed through the use of conventional chemical disinfectants, such as chlorine, chloramine and ozone (Richardson, Plewa, Wagner, Schoeny, & DeMarini, 2007). This fact, together with the rising demand for decentralized or point-of-use water treatment technologies, involves emerging technologies for efficient and safe water disinfection (Li et al., 2008).

Alternative technologies include UV radiation processes (Garvey, Hayes, Clifford, & Rowan, 2015; Hijnen, Beerendonk, & Medema, 2006), application of nanomaterials such as silver nanoparticles, titanium dioxide, chitosan or carbon nanotubes (Adeleye et al., 2016; Hossain, Perales-Perez, Hwang, & Román, 2014), and membrane filtration systems based on microfiltration, ultrafiltration or nanofiltration (Ang, Mohammad, Hilal, & Leo, 2015). UV radiation currently has its limitations, such as its ineffectiveness in reducing resistant microorganisms, high investment costs and potential health problems for people exposed to equipment's sources of irradiation (Pereira & Vicente, 2010; Sousa et al., 2017). Applying antimicrobial nanomaterials in drinking water treatments presents diverse problems, such as a potential impact on human health and the environment, preservation of antimicrobial activity, technical hurdles due to aggregation issues, regulatory challenges and the public's perception (Li et al., 2008). The application of membrane filtration has several limitations, such as fouling, significant energy

input to filter fluids through membranes and harsh cleaning requirements (Adeleye et al., 2016; Ang et al., 2015).

Filtration technologies can be improved through the immobilization of antimicrobial agents in filtering elements, which retain and inactivate microorganisms. Silver is an antimicrobial agent that has been embedded in different surfaces for water treatment to improve filter performance in order to inactivate coliform bacteria (Dankovich & Gray, 2011; Oyanedel-Craver & Smith, 2008). One example of the immobilization of natural antimicrobial agents is what Kroll et al. (2012) reported, that of anchoring a lysozyme to a porous ceramic filter. These authors obtained enhanced antibacterial properties for the microtubes functionalized with lysozyme for water treatment purposes.

Following this research approach, essential oil components (EOCs) were chosen herein as naturally-occurring antimicrobials to design new filtering elements. EOCs have been reported to exhibit antioxidant, antimicrobial, antifungal, antiviral and insecticidal activity (Hyldgaard, Mygind, & Meyer, 2012). Essential oils and their active compounds have been incorporated into different surfaces, such as film or paper, to be applied in active food packaging via impregnation (Mulla et al., 2017; Royo, Fernández-Pan, & Maté, 2010) or immobilization (Higueras, López-Carballo, Gavara, & Hernández-Muñoz, 2015). Recently, our research group reported a new antimicrobial system based on the covalent immobilization of EOCs on silica supports to preserve and enhance their antimicrobial effect against pathogen and spoilage microorganisms in both *in vitro* and *in situ* studies (Ribes et al., 2017; M. Ruiz-Rico et al., 2017). As a new application, the objective of this study was to evaluate these innovating supports as novel antimicrobial filtering elements for water treatment.

2. Materials and Methods

2.1 Chemicals

(3-Aminopropyl)triethoxysilane (APTES), paraformaldehyde, trimethylamine, 2-butanone, chloroform, glutaraldehyde, carvacrol (≥ 98% w/w), eugenol (99% w/w), thymol (≥ 98.5% w/w), sodium borohydride, KOH and silica particles (10, 25 and 50 μm) were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile, diethyl ether, dichloromethane, methanol, ethanol, n-hexane, NaCl, KCl, Na2HPO4, KH2PO4, MgSO4, H2SO4 and HCl were obtained from Scharlab (Barcelona, Spain). Vanillin (> 99% w/w) was supplied by Ventós (Barcelona, Spain). The amorphous silica particles (SYLYSIA® SY350/FCP, 5 μm) were acquired from Silysiamont (Milano, Italy).

2.2 Synthesis of EOC-functionalized supports

The EOC-functionalized silica microparticles (which are the supports) were synthesized following a four-step synthetic procedure (Ruiz-Rico et al., 2017). First, the aldehyde derivatives of carvacrol, eugenol and thymol were prepared to add a second reactive moiety to molecules to keep the hydroxyl group free, which is essential for bioactive compounds' antimicrobial activity (Gill & Holley, 2006). Second, the unmodified vanillin and previous synthesized aldehyde derivatives were reacted with APTES to obtain alkoxysilane derivatives capable of being attached to silica microparticles' surface in a third step. Finally, the imine bond of the alkoxysilane derivatives was transformed into an amine bond to stabilize the immobilized compounds. The detailed methodology is found in the Supplementary Material.

2.3 Materials characterization

Bare and EOC-functionalized supports were characterized by standard techniques to establish their morphology, surface charge and degree of

functionalization. The morphology of the bare and EOC-functionalized particles was characterized by field emission scanning electron microscopy (FESEM) using a Zeiss Ultra 55 microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) and were observed in the secondary electron mode. Surface charge was established by zeta potential (ζ-potential) analysis in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). For this purpose, particle suspensions in distilled water (1 mg/mL) were prepared and previously sonicated to prevent any aggregation of particles. The ζ-potential was calculated from the particle mobility values by applying the Smoluchowski model (Hunter, 1981). The degree of functionalization was determined by thermogravimetric 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 per min from 25 to 800 °C in an oxidant atmosphere (air, 80 mL/min).

2.4 Microbiological analysis

2.4.1 Reference strain and media

The bacterial strain used in the microbiological studies was the non-pathogenic strain of Escherichia coli K12 (CECT 433), obtained from the Colección Española de Cultivos Tipo (CECT; Valencia, Spain). This strain was chosen for its role as an indicator of fecal contamination to verify the drinking water's microbial quality (Tallon et al., 2005; World Health Organization, 2017). Plate Count Agar (PCA) and Tryptone Soy Broth (TSB) were used to grow E. coli K12. Peptone water (0.1%) was used to prepare decimal dilutions of the inoculum. The selective medium Tryptone Bile X-Glucuronide (TBX) agar was utilized to plate the microorganism in the antimicrobial assays. All the media were provided by Scharlab (Barcelona, Spain).

2.4.2 Preparing inoculated water

The bacterial strain was reconstituted following the CECT instructions and bacterial stock was stored at 4 °C in PCA before being used. The cells from an *E. coli* colony were transferred to a test tube with 10 mL of TSB to be incubated at 37 °C for 24 h to obtain an inoculum with an approximate microbial density of $1x10^9$ CFU/mL. Decimal dilutions of the inoculum were prepared in peptone water, while the last dilution was prepared in sterile distilled water to prepare Erlenmeyer flasks with 100 mL of water inoculated with *E. coli* (10^4 CFU/mL) by mixing the last decimal dilution with 90 mL of sterile distilled water.

2.4.3 Water filtration assays

As a first step, the water flow rate through the bed of untreated silica particles was determined as an important factor to technologically apply the developed supports. To this end, 100 mL of water were filtered through three layers of particles, whose thickness was 0.5, 1 or 1.5 cm in a filtration funnel (volume 250 mL, Ø 47 mm) to establish the amount of particles and the filtration time needed to obtain a 100 mL of permeate.

Four different filtration tests were used to evaluate the antimicrobial properties (removal capability of the filtration system with no loss of effectiveness, mechanism of action and potential leaching of the immobilized compounds) of the EOC-functionalized supports as filtering elements (Figure 1). First, 100 mL of inoculated water were filtered through the minimum thickness (0.5 cm) bed of the EOC-functionalized supports to assess the influence of particle size and immobilized EOC in the removal capability (Test 1). In order to clarify that the effect was to the covalently anchored EOCs and not to released EOCS, filters were pre-conditioned by filtering 1 L of sterile water. Then the removal properties of the EOC-functionalized particles were determined by filtering 100 mL of inoculated water (Test 2). In Test 3, three consecutive 100-mL inoculated water samples were filtered through the EOC-functionalized supports to assess their reuse capability. Finally,

the filtering supports' long-term efficiency was evaluated by using three subsequent pre-conditioning and filtering processes, for particles to be washed with 3 L of water (1 L per each pre-conditioning cycle) (Test 4). In all the 1-4 tests, three different particle thickness layers (0.5, 1 or 1.5 cm) were used with the 50-µm support given the inadequate results obtained with the thinnest layer.

The filtration assays were carried out using a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany) connected to an Erlenmeyer flask to collect the sample. After filtration, the collected water was plated in TBX and plates were incubated at 37 °C for 24 h. Viable cell numbers were determined as colony-forming units per mL (CFU/mL) with a detection limit of 5 CFU/mL. These values were logarithmically transformed and expressed as log CFU/mL.

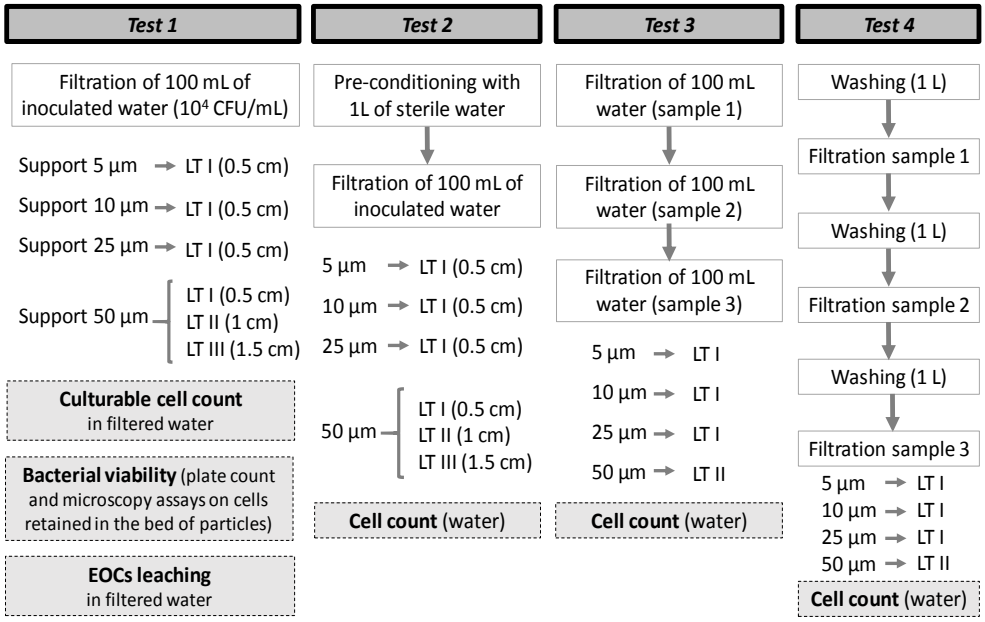

Figure 1. Summary of the water filtration assays performed to evaluate the microbial removal properties of the EOC-functionalized supports used as filtering materials. LT: layer thickness.

All the assays were performed in triplicate. Two control samples, non-filtered water and water filtered through a bed of bare silica particles, were included in the assays to quantify the microbial count in the absence of treatment and to then calculate the percentage of microbial reduction.

2.5 Determination of bacterial viability by culture count and microscopy assays

Plate counts were also conducted in the bed of particles (non-functionalized and EOC-functionalized supports) to evaluate the viability of the retained cells after performing Test 1. Particles were transferred to a stomacher bag after water filtration to be suspended in peptone water and incubated for 12 h at room temperature. Then decimal dilutions of the mixture were prepared and plated in media to assess the feasibility of the microbial cells retained in the bed of particles.

Two microscopy techniques were used to evaluate the viability of the cells retained in the filtration process by using the water filtered through the non-functionalized or functionalized with vanillin 10 µm-supports as reference samples (Test 1). The bed of particles was suspended in phosphate buffer and the mixture was stored for 3 h at room temperature. Then the LIVE/DEAD® BacLightTM kit (Life Technologies, Gaithersburg, MD, USA) was used to visualize viable (green) and dead (red) bacterial cells. For this purpose, 500 µL of suspension were mixed with 0.8 µL of the dyes (SYTO 9/propidium iodide, 1:1). After 10 min of incubation in the dark, 5 µL of mixture were placed on a poly-L-lysine-covered slide (Sigma-Aldrich, Madrid, Spain) and covered with a coverslip. Then bacterial viability was evaluated by microscopy observations under a Motic BA310E trinocular microscope equipped with an Epi-Led module, MB barrier filter and a Moticam 3+ camera.

FESEM was also used to evaluate the presence/absence of entrapped bacterial cells and their morphology after filtration. For the FESEM studies, the bacteria present in the bed of particles were fixed with 2% glutaraldehyde in phosphate buffer for 2 h at 4 °C, gradually dehydrated with ethanol 30, 50, 70, 80, 90, and 100%, and finally dried by the critical point method. A Zeiss Ultra 55 microscope

(Carl Zeiss NTS GmbH, Oberkochen, Germany) was used. Observations were made in the secondary electron mode.

2.6 Leaching the immobilized EOCs

Besides material characterization and the microbial removal tests, the potential leaching of the immobilized EOCs was evaluated by determining the presence of EOCs in filtered water. The quantification of compounds in water was made by gas chromatography-mass spectrometry (GC-MS). To do so, 100 mL of water were filtered through a bed of particles (5, 10, 25 or 50 µm). The EOCs released from the functionalized silica particles after filtering water were extracted with n-hexane and analyzed by GC-MS (Ribes, Fuentes, Talens, & Barat, 2016). A 5-mL aliquot of filtered water was mixed with 5 mL of n-hexane, and the mixture was gently shaken. After phase separation, the upper layer containing the EOCs was removed and transferred to a fresh vial, and the extraction process was repeated 3 times. The organic phase was evaporated under reduced pressure, and the obtained extracts were suspended in 2 mL of n-hexane and analyzed by GC-MS. GC-MS 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. The parameters for the MS analysis were EI Ion source, electron energy 70 eV, solvent delay 3 min and m/z 40-550 amu. EOCs were identified by matching the mass spectra with the standard mass spectra from the NIST MS Search 2.0 library and comparing the mass spectra of the pure compounds. EOCs were quantified according to the external standard method, in which a calibration curve of the peak area was used against the compound concentration.

2.7 Statistical analysis

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

3. Results and Discussion

3.1 Antimicrobial supports characterization

Sixteen supports functionalized with EOCs were prepared (four EOCs x four particle sizes) to evaluate their removal capability as filtering elements. Figure 2 shows the morphology of the bare and carvacrol-functionalized silica microparticles as an example of synthesized supports. The FESEM images show the size and shape of the different mean sized amorphous silica particles. In them the 5-µm particles present a sphere-like shaped irregular surface, while bigger supports display an irregular prism-shaped smooth surface. It is also worth highlighting that the immobilization process did not affect the structure of the different silica microparticles because the structure between the bare and functionalized supports was maintained.

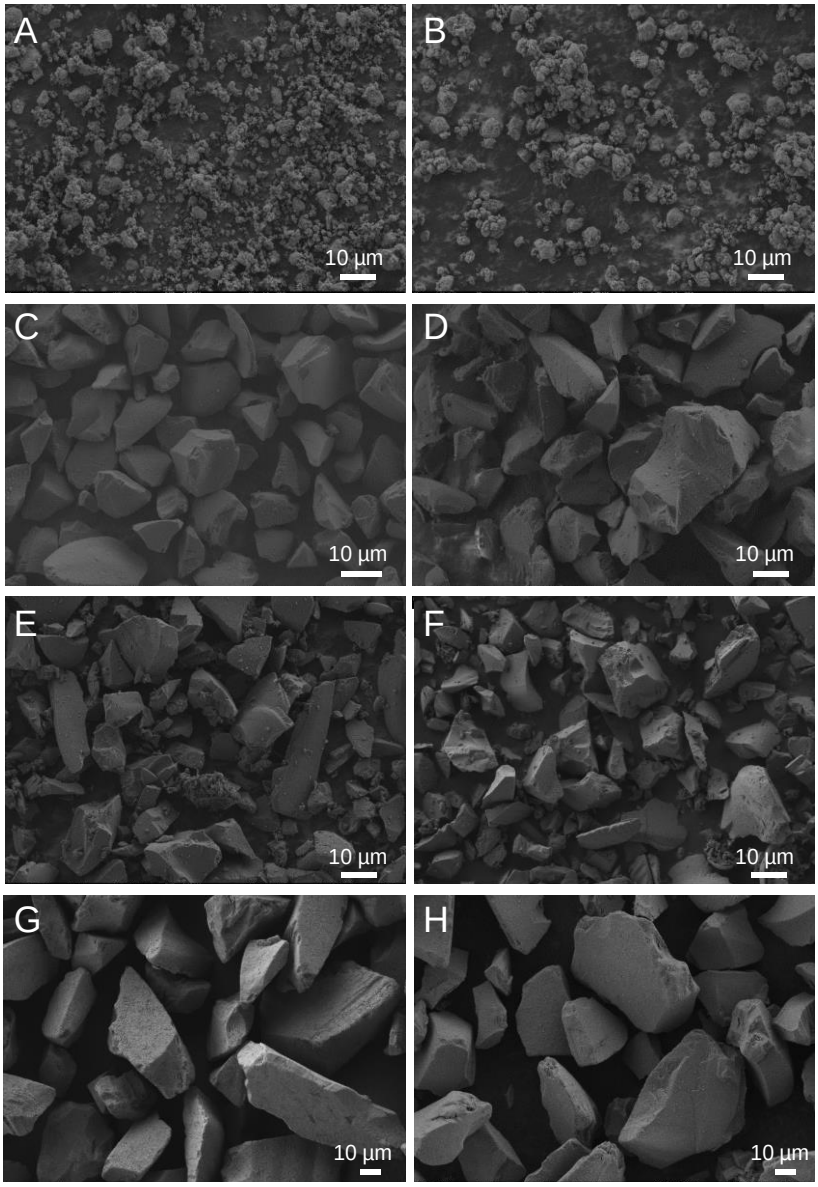

Figure 2. The FESEM images of the bare (A, C, E and G) and carvacrol-functionalized (B, D, F and H) silica microparticles with a mean particle size of 5 µm (A, B), 10 µm (C, D), 25 µm (E, F) and 50 µm (G, H).

Table 1 shows the surface charge of the developed supports determined by the ζ-potential. The bare particles exhibited negative zeta potential values, but the functionalized supports presented positive zeta potential values due to the attachment of the EOC-alkoxysilane derivatives. This change in surface charge confirmed that the bioactive compounds were attached to the particles' surface. Then the positively charged EOC-functionalized supports could show electrostatic attraction with the negatively charged bacterial surface to favor the supports' retention capability (Peña-Gómez, Ruiz-Rico, Fernández-Segovia, & Barat, 2018). Moreover, the analysis of the degree of functionalization by TGA determined the amount of organic matter to be ca. 0.01 g EOC/g SiO2 for carvacrol and thymol, 0.04 g EOC/g SiO2 for eugenol and 0.12 g EOC/g SiO2 for vanillin on the different supports.

Table 1. The 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	-30.6±0.8^{a}	23.3±1.1^{c}	14.8±1.8^{b}	27.5±0.6^{d}	29.8±0.9^{d}	***
10 µm	-23.4±2.7^{a}	12.8±3.8^{b}	32.2±0.8^{c}	15.7±1.2^{b}	25.0±1.9^{c}	***
25 µm	-33.8±1.1^{a}	19.3±4.1^{b}	40.0±1.1^{d}	16.3±0.5^{b}	24.2±0.2^{c}	***
50 µm	-29.1±6.8^{a}	14.9±2.5^{b}	36.5±3.1^{d}	27.6±5.4^{c}	28.6±1.1^{c}	***

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

3.2 Evaluating the microbial removal properties of the EOC-functionalized supports as filtering materials

3.2.1 Estimating flow parameters

As a preliminary study to evaluate the supports' removal capability, the key parameters used to technologically apply particles on a laboratory scale were determined (see Table S1). Water flow was faster for the supports with a larger mean particle size (25-50 µm), which required a bigger mass of particles to achieve

the same layer thickness, given their lower bulk density. In contrast, filtering through layer thickness II and III of 5-10-µm particles lasted several minutes, which indicates the influence of particle size on the flow of liquid through the bed. Prolonging the filtering time may favor the EOC-functionalized supports coming into contact with bacterial cells, which can be considered an advantage from the microbial safety point of view, but is a technological disadvantage to industrially apply the system.

Table S1. Mass of particles (g) and filtration time (min) for 100 mL of water according to particle size and bed thickness.

Bed thickness	Particle size (µm)	Solid (g)	Time (min)
Layer I (0.5 cm)	5	0.6	5
	10	2	3
	25	2	1
	50	2.5	0.1
Layer II (1 cm)	5	1.1	25
	10	4.4	18
	25	4	2
	50	6.7	0.2
Layer III (1.5 cm)	5	2.5	54
	10	7	27
	25	6.5	3
	50	12	0.5

3.2.2 Microbial removal capability of the EOC-functionalized supports after a single filtration

After evaluating the flow parameters, the antimicrobial properties of the developed supports were studied first by filtering the water inoculated with E. coli K12 (microbial density of 104 CFU/mL) through bare and functionalized particles. Filtering with bare particles slightly reduced the filtered water's microbial load up to 1-log cycle because most bacteria percolate through particles. The microbial count of the water filtered through the non functionalized supports was 2.9±0.5,

2.9±0.2, 3.1±0.1 and 3.3±0.1 log CFU/mL for supports with a mean particle size of 5, 10, 25 and 50 µm, respectively. The partial adsorption of bacterial cells in the bed of particles can explain the lower microbial count. Reducing E. coli using ceramic water filters has been previously described. Mineral pot filters, composed of porous ceramic filters and granular filtration with activated carbon, silica sand and zeolite, are used as household water treatment systems in developing countries. Karim et al. (2016) evaluated the effectiveness of these filters in reducing E. coli. These authors obtained an average reduction of 1.8-2.7 logarithmic cycles, which was insufficient to assure drinking water safety. Brady-Estévez, Kang and Elimelech (2008) reported a novel filter based on the immobilization of a single-walled carbon nanotube on a microporous ceramic filter for water treatment purposes. The results of the microscopic examination of the water permeates indicated that the bare base filter (5 µm pore size) allowed microbial cells to pass through the filter.

Figure 3 shows the microbial count of the inoculated water after filtering 100 mL of water through layer thickness I (0.5 cm) of the antimicrobial supports. The results revealed that the EOC-functionalized supports with a mean particle size of 5-25 µm displayed remarkable antimicrobial activity and reduced the microorganism to undetectable levels after a single filtration for most immobilized bioactive compounds. According to the statistical analysis, particle size and immobilized compound, and their interaction significantly impacted microbial reduction ($p<0.05$). The 5-25 µm-sized supports showed good bacterial removal capacity with log reduction values (LRVs) of ca. 4, which is an acceptable reduction for household water treatment technologies (LRV between 1-4 for granular media filters) according to the WHO guidelines for drinking water quality (World Health Organization, 2011).

In contrast, the largest supports (50 µm) showed less elimination capacity. The short contact time between the bacterial cells in water suspension and the immobilized bioactive compounds attached to particles (0.1 min for layer thickness I) could explain the significant microbial reduction differences compared to the smaller supports. For this reason, the removal properties assessment of layer

thicknesses II (1 cm) and III (1.5 cm) of the 50-µm supports was made, and achieved a greater microbial reduction of E. coli than with layer thickness I. In layer thickness II, the 50-µm support functionalized with carvacrol and thymol completely eliminated the microorganism in treated water, whereas microbial counts of 0.90±0.38 and 0.61±0.13 log CFU/mL were obtained for the eugenol and vanillin-functionalized supports, respectively. Total water microbial load removal was achieved after filtering through layer thickness III with all the supports. The enhanced retention capability of the 50 µm-supports by increasing bed thickness was probably due to the delayed filtration time favoring the contact between the microorganism and antimicrobial compounds.

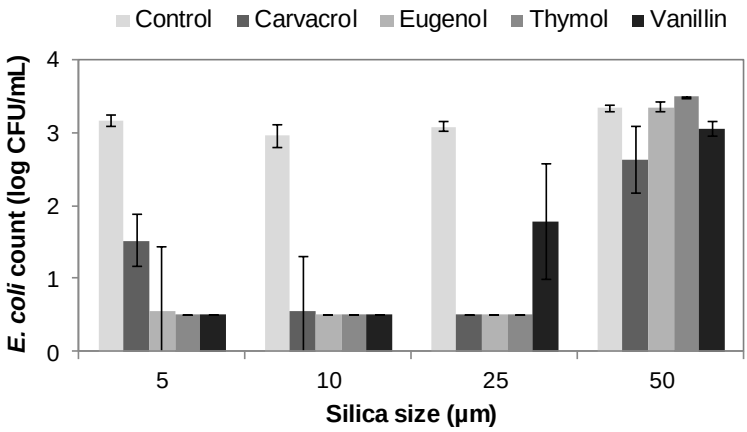

Figure 3. Microbial count of *E. coli* K12 (log CFU/mL) after filtering inoculated water through layer thickness I (0.5 cm) of the antimicrobial supports (means and standard deviations, n=3).

3.2.3 Antimicrobial properties of the EOC-functionalized supports after single filtration

Figure 4 shows the presence and viability of the retained bacterial cells in the bed of particles after a single filtration. As seen in Figure 4A, the non-filtered E. coli cells displayed the typical rod-shaped morphology with an intact cell envelope,

which was also confirmed by the abundance of green-colored cells in Figure 4B. After filtering through the non-functionalized support, retention of microbial cells in the bed of particles was observed in accordance with the results described in Section 3.2.1. The cells filtered with the non-modified particles showed unaltered cell walls and cell membranes given the morphology of the cells in Figure 4C and the presence of green cells adhered to particles in Figure 4D. In contrast, the filtration done with the vanillin-functionalized support clearly reduced bacterial viability given the absence of intact cells in the FESEM and fluorescence microscopy images (Fig. 4E-F).

Besides microcopy techniques, plate count was also conducted in the bed of particles to ensure that the antimicrobial activity only lay on EOCs. The microbial population retained by the bare particles continued to be feasible after incubation, whereas the incubation of the functionalized particles after filtration treatment displayed non-culturable bacterial cells in the support matrix (data not shown). Therefore, the bacterial removal of the filtration systems is due to the combination of physical adsorption and the inactivation by contact with the immobilized EOCs.

As mentioned above, physical adsorption can be favored by the electrostatic attraction between the negatively charged cell surface of microorganisms and the positively charged EOC-functionalized supports (Ruiz-Rico et al., 2018). To this the antimicrobial properties of EOCs against gram-negative microorganisms like E. coli must be added (Fitzgerald et al., 2004; Gill & Holley, 2006; Guarda, Rubilar, Miltz, & Galotto, 2011). The inhibitory effect of these bioactive compounds has been attributed to the presence of functional groups as the hydroxyl group, the relative position of this moiety in the phenolic ring, as well as the phenolic ring itself that has destabilized electrons. Thus the interaction of EOCs with the microbial cell envelope disturbs the cytoplasmic membrane and leads to intracellular components leaking, and eventually to cell death (Burt, 2004).

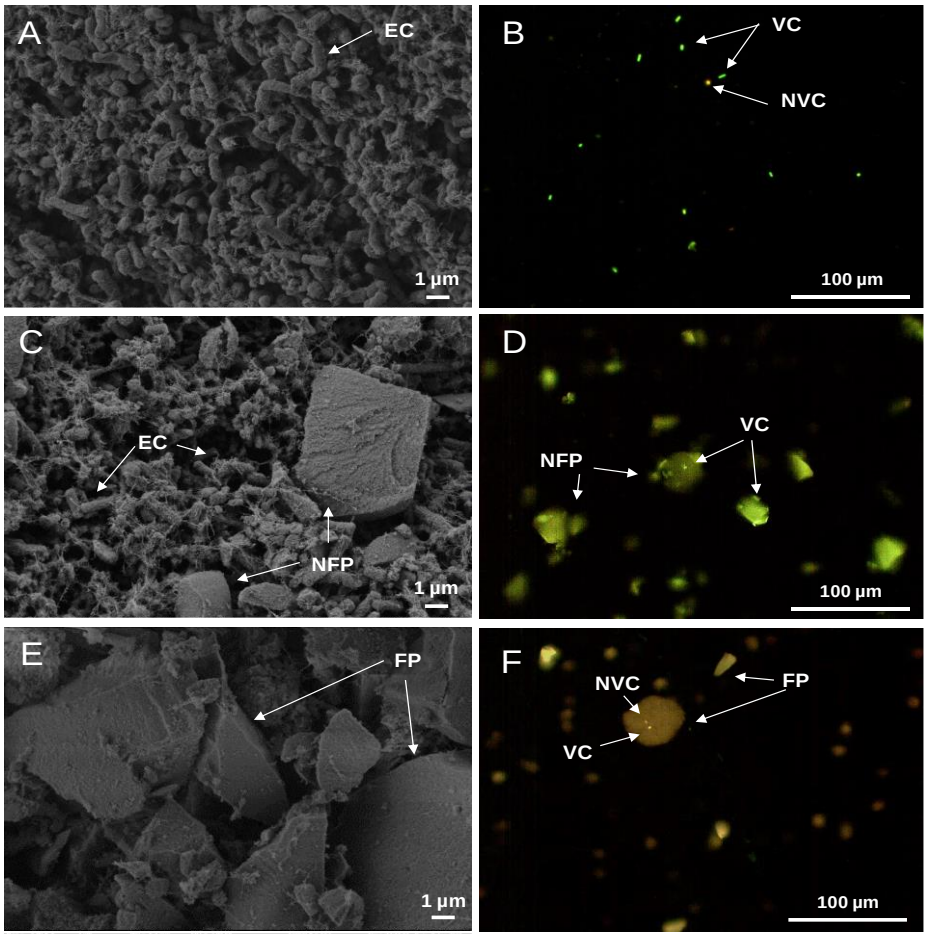

Figure 4. FESEM and fluorescence images of the non-filtered *E. coli* (A, B) and the cells retained in the bed of non-functionalized (C, D) and vanillin-functionalized support (E, F) after filtering 100 mL of water. FESEM observations were conducted with the bed of particles whereas fluorescence images were obtained from the particles suspended in phosphate buffer. EC: *E. coli* cells, VC: viable *E. coli* cells, green-colored; NVC: non-viable *E. coli* cells, red-colored; NFP: non-functionalized particles; FP: vanillin-functionalized particles.

The Test 1 results agree with some recent studies that have focused on developing antimicrobial particles for food applications. Ruiz-Rico et al. (2017) designed antimicrobial particles by the functionalization of mesoporous silica particles, fumed silica particles and amorphous silica particles with EOCs, and

studied their in vitro and in situ antibacterial activities against Listeria innocua and E. coli. The immobilization process greatly enhanced the anchored bioactive compounds' antimicrobial activity compared to free components after incubating in the presence of suspensions of the free or immobilized EOCs. Similarly, Ribes et al. (2017) reported the antimicrobial properties of eugenol and thymol immobilized on mesoporous silica particles against fungi development in vitro and in strawberry jam. The microbiological results showed improved immobilized EOCs' antifungal activity, and the sensory evaluation confirmed that the immobilization process reduced the impact of bioactive compounds on strawberry jam flavor.

3.2.4 Microbial removal capability of the EOC-functionalized supports after pre-conditioning

Figure 5 shows the microbial count after filtering 100 mL of inoculated water through layer thickness I of the bed of particles previously washed with 1 L of sterile water. The 5- and 10-µm supports well removed bacteria with as good a microbial reduction as in Test 1. The 25-µm functionalized particles inactivated the microorganism to undetectable levels for the different immobilized compounds. The results revealed that pre-conditioning with a high volume of water preserved, and even improved in some cases, the elimination properties of some EOC-functionalized particles. The retention capacity of the 50-µm supports was low after filtration through layer thickness I, but the water treatment with layer thicknesses II and III greatly enhanced their antimicrobial activity. Indeed the layer thickness II of the eugenol and vanillin-functionalized supports allowed E. coli population reductions of 0.53±0.17 and 0.70±0.23 log CFU/mL, respectively, whereas the filtration of immobilized carvacrol and thymol through layer thickness II resulted in total water decontamination. Washing and filtering through the layer thickness III of the 50 µm-supports completely retained bacteria. Similarly to the previous assay, the analysis of the Test 2 results by a multifactor ANOVA established the significant influence of the factors (particle size and type of immobilized EOC) on the microbial count variable ($p<0.05$). The fact that the antimicrobial supports continued to be

effective after the washing step confirmed the covalent immobilization of EOCs on the supports' surfaces by preventing the release of any attached bioactive compounds.

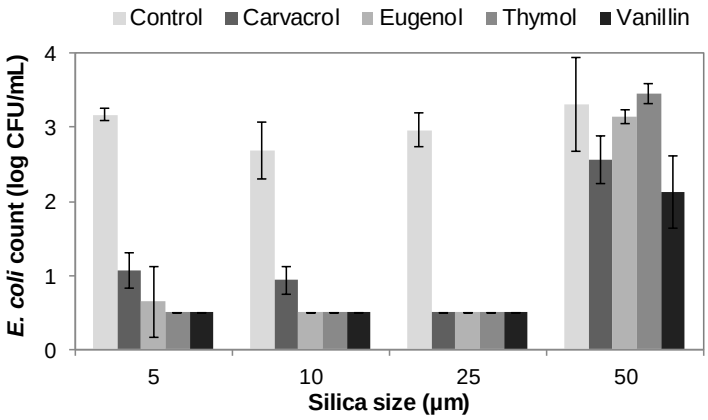

Figure 5. Microbial count of *E. coli* K12 (log CFU/mL) after filtering 1 L of sterile water and 100 mL of inoculated water through layer thickness I (0.5 cm) of the antimicrobial supports (means and standard deviations, n=3).

3.2.5 Microbial removal capability of the EOC-functionalized supports after pre-conditioning and multiple filtrations

After establishing the disinfection potential of the EOC-functionalized particles by a single filtration, the EOC-functionalized supports reuse capability as filtering elements was evaluated by filtering three consecutive water samples (Test 3), and by pre-conditioning followed by filtering three samples (Test 4).

Figure 6 shows the E. coli counts after successive filtrations through the same bed of particles. For the 5-µm supports (Fig. 6A), the inhibitory effect progressively enhanced after consecutive filtrations and complete microorganism elimination was achieved for the third filtration, except for the carvacrol-functionalized particles. For the 10-µm supports (Fig. 6B), good removal capability was preserved after treating multiple water samples. Likewise, the 25-µm functionalized particles

(Fig. 6C) showed good bacterial elimination after treating multiple samples, except for the immobilized vanillin. By considering the Test 1 and 2 results, the reuse capability test was carried out for the layer thickness II of the largest supports (50 µm). As seen in Figure 6D, the supports' thicker layer displayed complete microorganism elimination for carvacrol, eugenol and thymol, whereas the vanillin-functionalized particles achieved a mean 2-log E. coli reduction for the three filtered water samples.

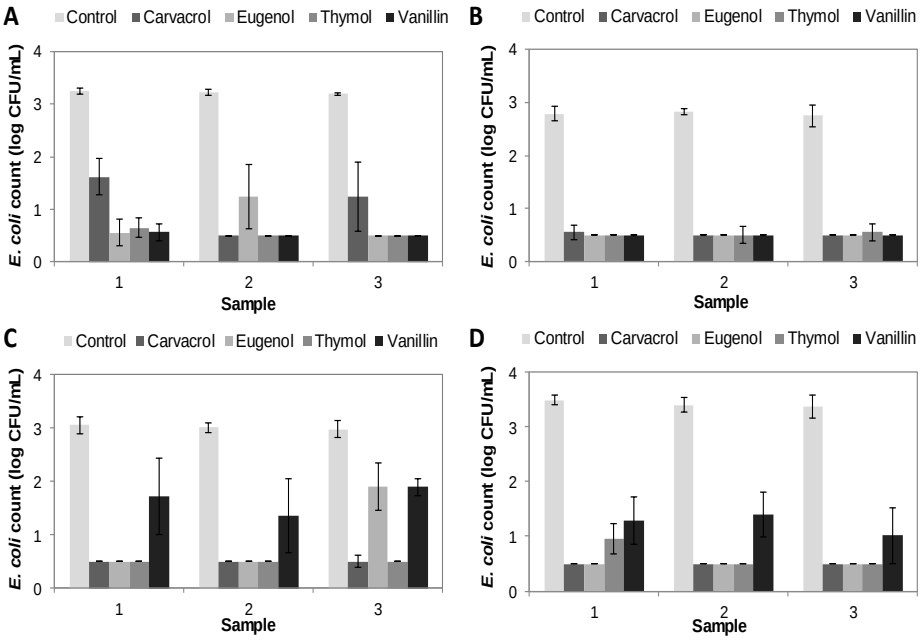

Figure 6. Microbial count of *E. coli* K12 (log CFU/mL) after filtering multiple samples of inoculated water through the antimicrobial supports. Layer thickness I (0.5 cm) of particles of a mean size of 5 µm (A), 10 µm (B) and 25 µm (C) and layer thickness II (1 cm) of the particles of 50 µm (D) (means and standard deviations, n=3).

Figure S1 illustrates the removal potential of the antimicrobial supports after filtering multiple samples by interspersing supports' pre-conditioning with 1 L of

sterile water. The pre-treatment with sterile water improved the EOC-functionalized particles' removal capacity, except for the 10-µm support that obtained heterogeneous results. The supports' enhanced inactivating effect after washing could be due to the compaction of the particle bed and the consequent increased filtration time, which would favor the contact between the microorganism and the attached bioactive compounds.

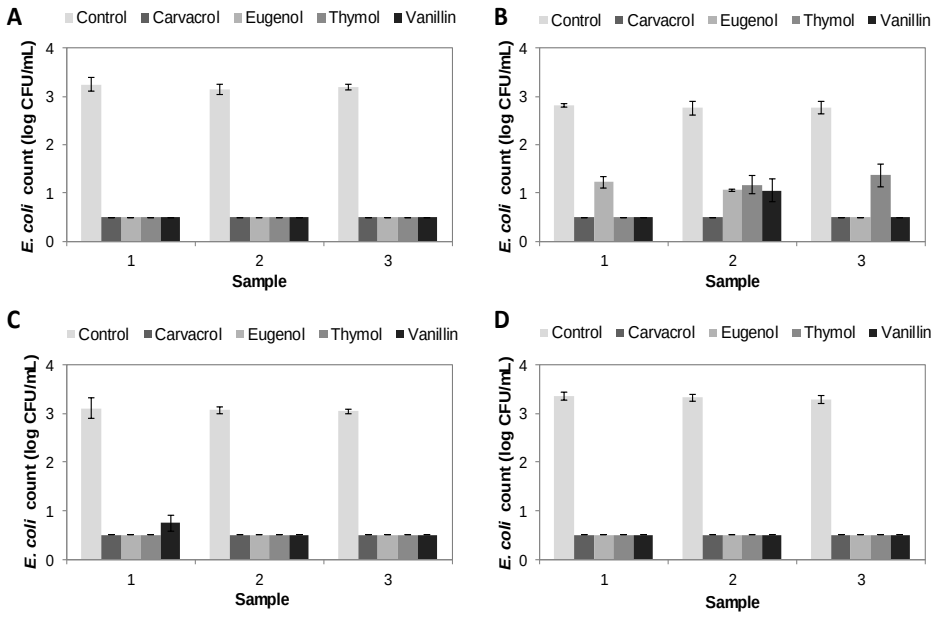

Figure S1. Microbial count of *E. coli* K12 (log CFU/mL) after filtering 1 L of sterile water between the filtrations of multiple inoculated water samples through the antimicrobial supports. Layer thickness I (0.5 cm) of particles of a mean size of 5 µm (A), 10 µm (B) and 25 µm (C) and layer thickness II (1 cm) of the particles of 50 µm (D) (means and standard deviations, n=3).

The results obtained in a washing step also confirmed the supports' removal properties being preserved after passing through a high water volume (3 L in total). This fact suggests the stable immobilization of the bioactive compounds onto the

supports' surface, which would hinder the release of or lixiviate the attached molecules, which contrasts with some results reported about antimicrobial surfaces, where antimicrobial agents were embedded on the surface with loss after the filtration procedure (Dankovich & Gray, 2011; Kroll et al., 2012; Oyanedel-Craver & Smith, 2008).

A multifactor ANOVA analysis was performed with the Test 3 and 4 results to explain the influence of the different factors on the E. coli counts after multiple filtrations (Table 2). This analysis confirmed the significant impact of the factors (number of filtered sample, particle size and immobilized compound), as well as the interaction between factors, on the bacterial counts, similarly to the above-described results.

The fact that the supports' removal capacity remained after washing and filtering multiple samples with at least 4 LRVs confirms the stability of the immobilized EOCs on the silica supports and their potential application as a water treatment technology.

Table 2. The F-ratio values and significance levels obtained in the multifactor ANOVA for the different factors and their interaction in the retention of *E. coli* K12 after filtering three consecutive samples with or without pre-conditioning.

	Multiple filtration		**Multiple filtration + pre-conditioning**	
Factor	**F-ratio**	**α**	**F-ratio**	**α**
Particle size	7.19	***	39.88	***
EOC	8.77	***	6.44	***
Nº filtered sample	1.92	ns	3.69	*
Particle x EOC	10.88	***	7.64	***
Particle x Nº sample	3.65	**	6.13	***
EOC x Nº sample	1.54	ns	5.03	***
Particle x EOC x Nº sample	2.52	**	5.75	***

Significance level (α): ns: non-significant, * p<0.05, ** p<0.01, ***p<0.001

3.3 EOCs leaching

To evaluate the stability of the immobilized EOCs on silica supports and to determine the wash-out effects under our filtration conditions, the potential leaching of the attached bioactive compounds was quantified. By taking into account the total attached EOCs to the supports, as determined by the TGA and elemental analyses (Section 3.1) and the amount of particles needed to prepare the bed of particles, the percentage of leached compounds was calculated after analyzing the filtered water.

Table 3 presents the amount of lixiviated EOCs and the percentage of leached compounds in the effluent water after passing 100 mL water through the bed of particles. In general, no wash-out effects after filtration resulted in the stable EOCs bonding on the supports' surface for eugenol, thymol and vanillin (values below the detection limit). In contrast, the water filtered through the carvacrol-functionalized supports showed a carvacrol content in the effluent water, which was 4.9-9.6% of the initial EOCs' content of particles. The release of this compound from the supports' surface could be due to problems while preparing the supports (incorrect immobilization process or washing).

The quantification of the EOCs' leaching demonstrated that covalent immobilization preserved the EOCs' attachment and the longevity of the filtration technology in accordance with the bacterial removal tests. The absence, or even the presence, of the EOCs that are considered GRAS at low concentrations (Hyldgaard et al., 2012) in the treated water ensures the safety of the filtration technology given the possibility of the repeated or continuous reuse of the immobilized naturally-occurring antimicrobials and the prevention of toxic or carcinogenic disinfection byproducts, which derive from using conventional chemical disinfectants (Richardson et al., 2007). Nevertheless, the immobilization procedure should be optimized to achieve a zero release of the attached molecules.

Table 3. EOCs leaching (mg) and the relative percentage of leached EOC after filtering 100 mL of water through the bed of the EOC-functionalized supports. Mean values ± SD (n=3).

Size	Carvacrol	Eugenol	Thymol	Vanillin
5 µm	0.6±0.1 (9.6%)	nd (0.0%)	nd (0.0%)	1.3±0.1 (1.9%)
10 µm	1.0±0.1 (4.9%)	0.8±0.1 (1.0%)	0.8±0.0 (3.8%)	nd (0.0%)
25 µm	1.2±0.4 (6.2%)	nd (0.0%)	nd (0.0%)	nd (0.0%)
50 µm	nd (0.0%)	nd (0.0%)	nd (0.0%)	nd (0.0%)

nd (no detected)

4. Conclusions

The EOC-functionalized supports developed herein displayed disinfection properties against E. coli K12 in filtered water. Therefore, the filtration process based on carvacrol, eugenol, thymol, and vanillin immobilized on silica microparticles, developed as a proof of concept, displayed a very high potential to be used as a novel water treatment, and has the potential to be used for treatment of liquid food like juice, milk, beer or wine. Moreover, silica supports functionalized with carvacrol, eugenol, thymol, and vanillin can be used to design novel small-scale or point-of-use systems to increase the robustness of water supply networks or to treat water in areas with water shortage problems by showing a high bacterial removal capacity with 4 LRV following WHO requirements. However, before it is applied to a real scenario, it is necessary to study the removal capability against different pathogenic microorganisms and to evaluate the potential synergic effect of combining different functionalized supports.

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

Nataly Peña-Gómez[a], María Ruiz-Rico[a]*, Édgar Pérez-Esteve[a], Isabel Fernández-Segovia[a], José M. Barat[a]

[a]Grupo de Investigación e Innovación Alimentaria. Departamento de Tecnología de Alimentos, Universitat Politècnica de València. Camino de Vera s/n, 46022, Valencia, Spain

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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

1.1. 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, MgSO4, H2SO4 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.

1.2. 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.

1.3. 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).

1.4. 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.

1.5. 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.

1.6. 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.

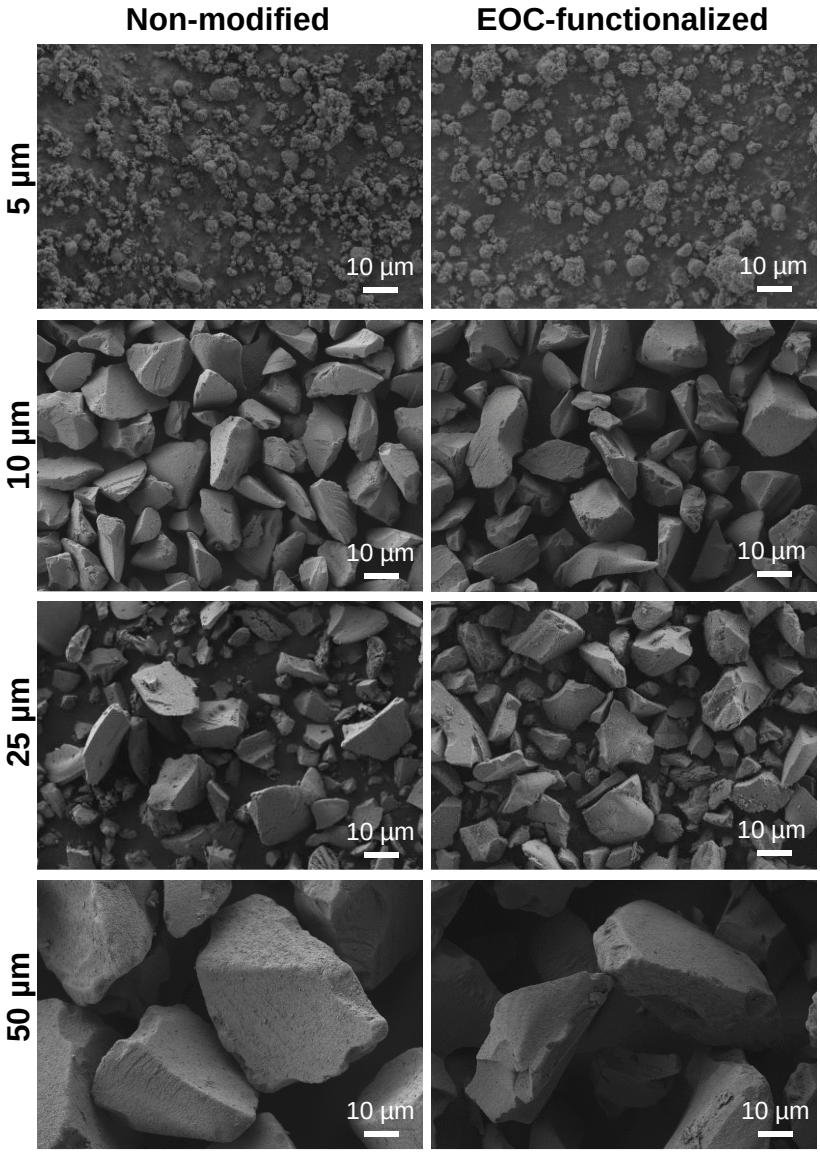

Figure 1. Field emission scanning electron microscopy images of the bare and carvacrol-functionalized silica microparticles.

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*.

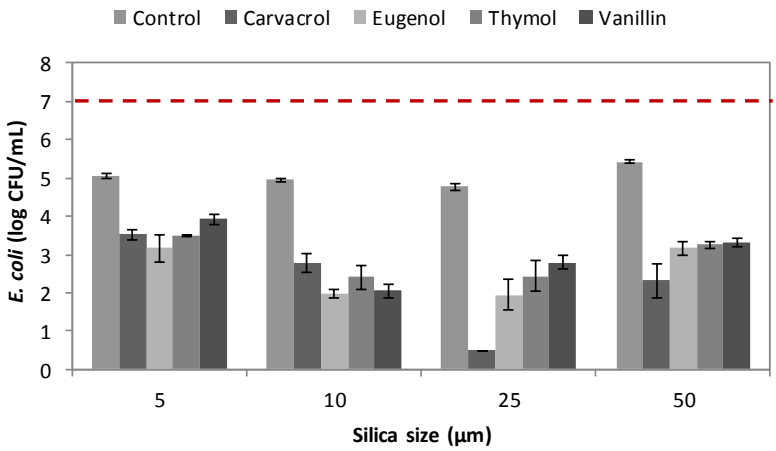

Figure 2. Microbial counts (log CFU/mL) of *E. coli* inoculated in commercial pasteurized beer after filtering beer with non-functionalized particles (control) and EOC-functionalized supports. Mean values ± SD (n=3).

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.

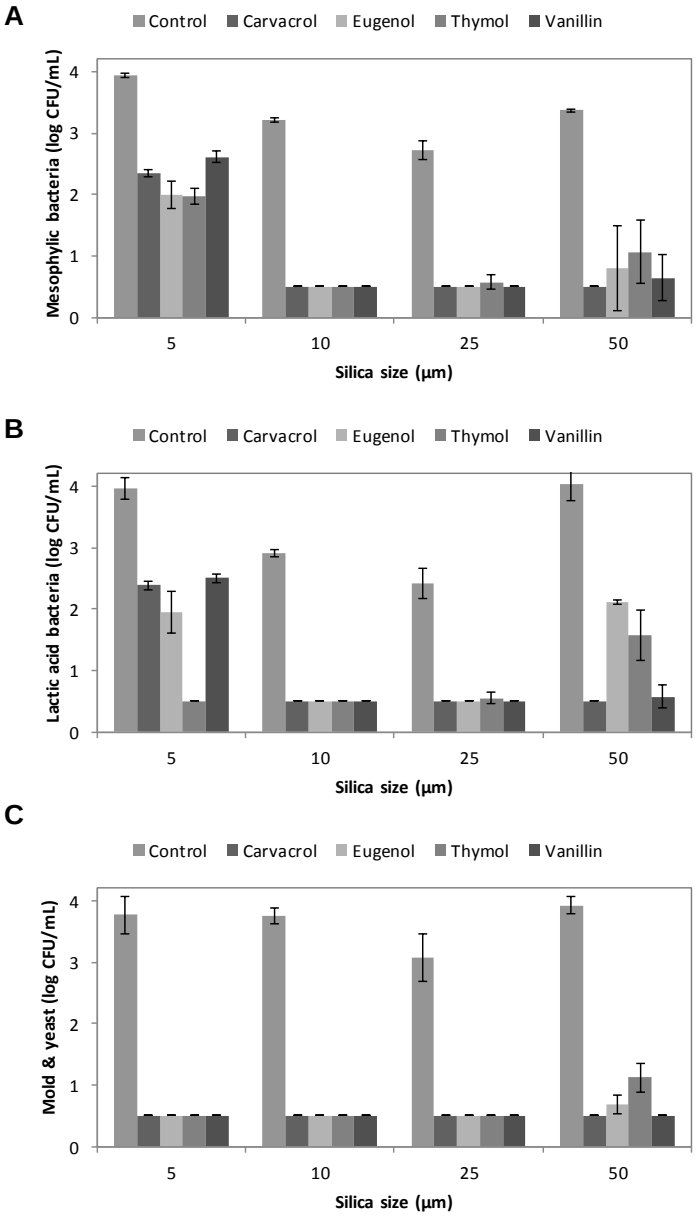

Figure 3. Microbial counts (log CFU/mL) of mesophilic bacteria (A), lactic acid bacteria (B) and mold and yeast (C) after filtering beer with non-functionalized particles (control) and EOC-functionalized supports. Mean values ± SD (n=3).

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.

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 ($p<0.01$). 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.

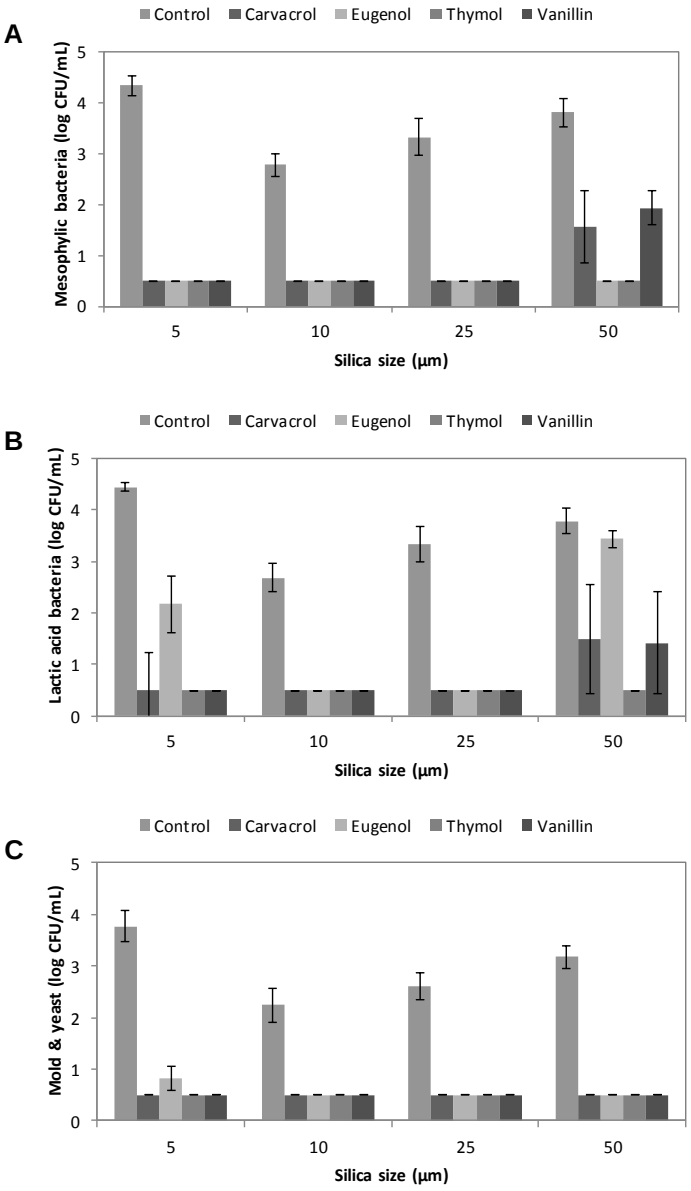

Figure 4. Microbial counts (log CFU/mL) of mesophilic bacteria (A), lactic acid bacteria (B) and mold and yeast (C) after pre-washing and filtering beer with non-functionalized particles (control) and EOC-functionalized supports. Mean values ± SD (n=3).

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**
5 µm	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
	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
10 µm	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	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
25 µm	1	nd	nd	0.6 ± 0.2	nd	nd	nd	0.6 ± 0.2	nd	nd	nd	nd	nd
	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
50 µm	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
	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**
5 µm	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	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
10 µm	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	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
25 µm	1	nd	nd	nd	nd	nd	nd	nd	0.6 ± 0.1	nd	nd	nd	nd
	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
50 µm	1	1.6 ± 0.6	nd	nd	1.1 ± 0.6	nd	0.9 ± 0.2	nd	nd	nd	nd	nd	nd
	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)

3.3. 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.

Table S1. EOCs leaching (mg) and the relative percentage of leached EOC after filtering beer through the bed of the EOC-functionalized supports. Mean values ± SD (n=3).

Size	**Carvacrol**	**Eugenol**	**Thymol**	**Vanillin**
5 µm	nd (0.0%)	nd (0.0%)	nd (0.0%)	nd (0.0%)
10 µm	nd (0.0%)	nd (0.0%)	nd (0.0%)	nd (0.0%)
25 µm	nd (0.0%)	nd (0.0%)	nd (0.0%)	nd (0.0%)
50 µm	nd (0.0%)	nd (0.0%)	nd (0.0%))	nd (0.0%)

nd (no detected)

3.4. 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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6.3. Study of apple juice preservation by filtration through silica microparticles functionalized with essential oil components

Nataly Peña-Gómez[a], María Ruiz-Rico[a], Isabel Fernández-Segovia[a], José M. Barat[a]

[a]Grupo de Investigación e Innovación Alimentaria. Departamento de Tecnología de Alimentos, Universitat Politècnica de València. Camino de Vera s/n, 46022, Valencia, Spain

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Abstract

Novel non-thermal preservation technologies are needed given consumers' demand for minimally processed high quality food products with a long shelf life. In this work, novel filtering materials based on silica microparticles functionalized with essential oil components (eugenol and vanillin) were applied as an alternative preservation method for apple juice. After confirming the immobilization of the antimicrobial compounds on particles' surface, the capacity of the filtration system to pasteurize apple juice inoculated with *Escherichia coli* was proven (a reduction of at least 5-log). The influence of filtration on the physicochemical (pH, acidity, soluble solids, color) and microbiological (mesophilic, psychrophilic and mold and yeast) parameters of fresh apple juice was evaluated throughout refrigerated storage, as well as the potential leaching of the immobilized bioactive compounds. The results showed the capability of the functionalized particles to completely remove the native flora of fresh apple juice and to preserve the product's microbial stability throughout the study period (> 120 days). Filtering juice through the eugenol-functionalized particles did not strongly affect the physicochemical properties, except for the color attribute. The use of immobilized vanillin significantly affected some product characteristics, probably due to the partial release of compounds to the food matrix. Therefore, the immobilization methodology should be optimized before applying this support to a real scenario. Nevertheless, the immobilization of these bioactive compounds on silica microparticles allowed filtration technology to be developed as a promising alternative to existing pasteurization technologies to provide microbial stable and quality acceptable food products.

Keywords: naturally-occurring antimicrobial compounds; cold pasteurization; covalent immobilization; filtration; silica microparticles; apple juice.

1. Introduction

Apple juice can be contaminated by certain pathogens, such as *Escherichia coli* O157:H7, *Salmonella enterica* or *Cryptosporidium parvum,* given their ability to adapt to cooling temperatures and low pH. Therefore, the consumption of fresh apple juice has been linked to various outbreaks of diarrhea and hemolytic uremic syndrome (Martínez-Gonzáles & Castillo, 2016). In addition to pathogenic microorganisms, juice can also contain altering microorganisms, such as yeasts, mainly *Saccharomyces spp*., and molds like genera *Penicillium* and *Aspergillus,* and lactic acid bacteria like *Lactobacillus* and *Leuconostoc spp*. (Choi & Nielsen, 2005; Ferrario & Guerrero, 2016). Juice contamination is prevented by pasteurization methodologies, done to ensure a 5-log reduction in the microorganisms that can cause spoilage and pose public health problems, according to the US FDA (Food and Drug Administration, 2001).

Traditionally, thermal pasteurization has been the gold standard of preservation treatment. Thermal processing involves heating up food, which results in some degree of nutritional loss, undesirable sensorial changes and loss of some functional properties (Choi & Nielsen, 2005). Due to the negative effect of thermal pasteurization on key juice parameters and the growing consumer demand for minimally processed foods, alternative non-thermal pasteurization techniques have appeared in recent years. These methods should ensure that pathogenic microorganisms are absent, and they should prevent the growth of spoilage microorganisms from developing to extend the product's shelf life and to guarantee a minimum impact on nutritional and sensory food properties. New non-thermal technologies, such as pulsed light, ultraviolet radiation, ultrasound, high hydrostatic pressure, pulsed electric field and dense phase carbon dioxide, have been developed. However, they imply several limitations that prevent their industrial application, including limited antimicrobial efficacy (Chemat, Zill-E-Huma, & Khan, 2011), impact on food properties (Ahmed & Ramaswamy, 2003), and high implementation costs (Morris, Brody, & Wicker, 2007).

Filtration is an important non-thermal process used for the clarification, concentration and microbial stabilization of different foods (Papafotopoulou-Patrinou et al., 2016). Deep-bed filtration using sand or diatomaceous earth is used to eliminate organic matter and microorganisms, but does not offer sufficient efficiency and presents regeneration problems (Devi, Alemayehu, Singh, Kumar, & Mengistie, 2008). Membrane filtration offers selective filtration that reduces microbial contamination without having to use heat treatment (Lipnizki, 2010). However, the retention of food components, membrane fouling and cleaning requirements are critical factors that limit the extensive application of this technology (Fuenmayor, Lemma, Mannino, Mimmo, & Scampicchio, 2014).

Filtration as a methodology is continuously progressing and alternative materials can be proposed to design new filtration systems to be used as a food preservation technology. Among these novel materials, it is worth highlighting supports functionalized with antimicrobial molecules. Essential oil components (EOCs), as natural antimicrobials with recognized antimicrobial and antifungal activity (Burt, 2004), can be immobilized on silica supports to create new antimicrobial particles, and to prevent the negative effects associated with their use in a free form (strong smell and taste, high volatility or instability) (Tyagi, Gottardi, Malik, & Guerzoni, 2014). The inhibitory effect of immobilized EOCs has been recently demonstrated *in vitro* and in food matrices (Ruiz-Rico et al., 2017). Immobilization of EOCs on silica particles has provided improved antimicrobial properties of bioactive compounds (García-Ríos, Ruiz-Rico, Guillamón, Pérez-Esteve, & Barat, 2018) with a significantly reduced impact on aromas of treated foods (Ribes et al., 2017).

By taking these antimicrobial supports as starting point, this work aims to apply EOC-functionalized silica microparticles as filtering aids for the cold pasteurization of fresh apple juice, and to evaluate the influence of the filtration process on the physicochemical and microbiological properties of apple juice during refrigerated storage.

2. Materials and Methods

2.1. Chemicals

Eugenol, (3-aminopropyl)triethoxysilane (APTES), sodium borohydride, sodium hydroxide, chloroform, n-butanone and silica microparticles (mean size of 50 µm) were provided by Sigma-Aldrich (Madrid, Spain). Vanillin was purchased from Ventós (Barcelona, Spain). Acetonitrile, n-hexane, methanol, potassium hydroxide, sulphuric acid, dispersive solid phase Extrabond® QuEChERS and microbiological media were provided by Scharlab (Barcelona, Spain).

2.2. Synthesis of the EOC-functionalized silica microparticles

Antimicrobial supports were prepared by the covalent immobilization of vanillin and eugenol on the surface of commercial silica microparticles following the synthetic procedure of García-Ríos et al. (2018). For eugenol, the aldehyde derivative was prepared to add a second reactive moiety in the molecule. The aldehyde derivative of eugenol and unmodified vanillin were reacted with APTES to obtain alkoxysilane derivatives capable of being attached to silica microparticles' surface. Finally, the imine bond formed between the aldehyde group of bioactive compounds and the amino group of the alkoxysilane moiety was reduced to stabilize the immobilized antimicrobial compounds.

2.3. Materials characterization

The commercial silica microparticles and the EOCs-functionalized supports were characterized by standard techniques. Zeta potential analysis was performed in a Zetasizer Nano ZS (Malvern Instruments, UK) using previously sonicated particle suspensions in distilled water (1 mg/mL). Zeta potential values were obtained by applying the Smoluchowski model. The degree of functionalization was determined by thermogravimetric (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°C to 1,000°C in an oxidant atmosphere (air, 80 mL/min).

2.4. Assessment of the EOCs-functionalized supports as filtering materials for juice cold pasteurization

To test the effectiveness of the EOC-functionalized supports used as filtering materials to microbiologically stabilize juices, the preservation method should be able to reduce by at least 5 logarithmic cycles of pathogenic microorganisms to ensure the safety of the treated product (Kiskó & Roller, 2005). Therefore, this assay was performed using commercial juice inoculated with *Escherichia coli.* The non-pathogenic strain *Escherichia coli* K12 (CECT 433, Colección Española de Cultivos Tipo, Valencia, Spain) was used as a surrogate of *E. coli* O157:H7, which can mimic the pathogen's survival and growth properties (Kim & Harrison, 2009). Tryptic soy broth (TSB) and plate count agar (PCA) were used to grow the bacterium. The bacterial strain was reconstituted following the CECT instructions and bacterial stock was stored at 4°C in PCA before being used. To obtain an inoculum with an approximate microbial density of 10^9 cells/mL, the cells from a colony were transferred to a test tube with 10 mL of TSB to be incubated at 37°C for 24 h. After incubation, the cell concentration of the inoculum was checked by determining optical density at 600 nm in a Helios Zeta UV-VIS instrument (Thermo Scientific, Hampton, New Hampshire, USA). Then, the inoculum was centrifuged at 4,000 rpm for 10 min and the precipitated cells were resuspended in 1 L of apple juice to obtain a final *E. coli* density of 10^6 cells/mL.

The filtration assays were performed in a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany) connected to an Erlenmeyer flask to collect the sample. The filtering bed placed in the funnel consisted of three layers: (i) cellulosic paper on the manifold base; (ii) a bed of bare EOCs-functionalized supports (layer of 0.5-1 cm thickness); (iii) another cellulosic membrane as the covering top. For the filtration tests, the microbial retention

capability of the EOCs-functionalized supports was assessed after filtering 100 mL of inoculated apple juice through the bed of particles. Then the collected apple juice was plated in selective media Tryptone Bile X-glucuronide (TBX) agar, and plates were incubated at 37°C for 24 h. The values of the counts were logarithmically transformed and expressed as $\log_{10}$ CFU/mL.

Assays were performed in triplicate. Two control samples, namely the non-filtered juice and the juice filtered through a bed of bare (non-functionalized) silica particles, were included in the assays to quantify the microbial count in the absence of treatment and after filtration through bare supports.

2.5. Influence of the filtration process on the shelf life of fresh apple juice

After establishing the capability of the EOCs-functionalized supports as filtering elements to cold-pasteurize commercial inoculated juice, the influence of the filtration process on the different physic-chemical and microbiological parameters of apple juice during refrigerated storage was evaluated. Besides, the potential leaching of the immobilized molecules and the influence of the treatment on the sensory properties after filtration were determined.

2.5.1. Apple juice preparation

Apple juice was prepared with 'Braeburn' apples (*Malus domestica)*. After purchasing apples in a local supermarket, juice was prepared in a Thermomix TM31 (Vormerk MSL, Madrid, Spain), where the peeled and chopped apples were mixed with distilled water (1 L of water per kg of apples). The mixture was filtered through a stainless steel strainer without pressure to eliminate fruit pulp and to obtain fresh juice.

Fresh apple juice (150 mL) was filtered under sterile conditions through the layer (0.5 cm thickness, 2 g) of the bare or EOCs-functionalized supports and was stored in proportions of 20 mL in Falcon tubes at 4°C to be analyzed at different

times. The assay was done in triplicate, including the above-mentioned control samples.

2.5.2. Analytical methods for shelf life evaluation

Different analytical determinations were carried out during the shelf life of both fresh juice and filtered and refrigerated juice. The microbial analyses were carried out on days 0, 3, 6, 9, 12, 30, 60, 90 and 120. The physic-chemical parameters were determined on days 0, 3, 6, 9 and 12. All the analyses were performed in triplicate.

The microbial analyses consisted of enumerating the native flora of apple juice, including mesophilic aerobic, psychrophilic and mold and yeast. Peptone water 10-fold dilution aliquots of juice were prepared and plated on selective media according to the target microorganism. For the enumeration of the mesophilic aerobic populations, samples were plated in PCA to be incubated at 30°C for 72 h. The psychrophilic bacteria were determined on PCA plates incubated at 4°C for 10 days. The mold and yeast populations were counted on potato dextrose agar plates and incubated at 25°C for 72 h (Ferrario & Guerrero, 2016). The CFU/mL were determined (detection limit of 5 CFU/mL) and were logarithmically transformed and expressed as log CFU/mL.

The determined physic-chemical parameters were titratable acidity, pH, °Brix and color. An acidity analysis was done by titration with NaOH (0.1 N) in the presence of phenolphthalein. A pH-meter Crison micropH 2001 (Crison Instruments S.A., Alella, Barcelona, Spain) was used to measure the samples' pH value. Soluble solids (°Brix) determination was made with a refractometer RFM300 (Bellingham & Stanley Ltd, Kent, UK). Juice color was measured by a colorimeter Minolta CM 3600D (Minolta, Osaka, Japan) under a standard illuminant D65 and 10° observer. The CIEL*a*b* uniform color space was selected to calculate the color parameters: L* (brightness), a* (red–green) and b* (yellow–blue). The total color difference between the unfiltered sample and the samples treated on day 0 was also calculated.

2.5.3. Leaching the immobilized EOCs

Besides the microbiological and physic-chemical parameters, the possible leaching of the immobilized bioactive compounds was determined by gas chromatography-mass spectrometry (GC-MS). The QuEChERS procedure was followed for the extraction of EOCs based on an extraction phase with an organic solvent and different salts, and a clean-up step of the organic extract by dispersive solid-phase extraction (d-SPE). Vanillin was extracted from the juice samples (5 mL) using 10 mL of acetonitrile in the presence of sodium chloride and magnesium sulphate. After vigorous stirring, samples were centrifuged for 3 min at 3,300 rpm. The organic layer was transferred to the d-SPE tube (PSA and magnesium sulphate). The tube was vortexed for 1 min and centrifuged at 3,300 rpm for 3 min. The liquid extract was collected and evaporated under reduced pressure. Finally, the obtained extracts were suspended in 2 mL of methanol and were analyzed by GC-MS.

The analysis was performed in a 6890/5975 inert GC-MS (Agilent Technologies, USA), equipped with an 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 before being 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. The parameters for the MS analysis were the EI Ion source, electron energy 70 eV, solvent delay 3 min and m/z 40-550 amu. EOCs were identified by matching the mass spectra with the standard mass spectra from the NIST MS Search 2.0 library and comparing the mass spectra of the pure compounds. EOCs were quantified according to the external standard method, in which a calibration curve (0.5, 1, 5, 10, 25, 50, 100, 250 and 500 mg/L) of the peak area was used against the compound concentration (Ribes, Fuentes, Talens, & Barat, 2016).

2.5.4. Sensory evaluation

A sensory test was run to determine the consumer acceptability of the apple juice filtered through the eugenol-functionalized supports as the most effective filtering material in accordance with the results obtained with previous analyses. The juice filtered through the non-functionalized particles was also included as the control samples. The panel involved 44 untrained panelists (29 females, 15 males), whose ages ranged from 21 to 48 years. Four sensory parameters (appearance, color, odor and general acceptance) were judged on a 9-point hedonic scale (1 = very much dislike, 9 = very much like) (UNE-ISO 4121:2003). Judges also evaluated the typical odor of juices on a 5-point hedonic scale (1 = not typical, 5 = very typical). Individual juice samples (10 mL) were presented to the panelists at room temperature in a capped transparent glass phial with random 3-digit code.

2.6. Statistical analysis

Data were statistically analyzed using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). The effect of filtration and storage time on the physic-chemical and microbiological parameters of apple juice was analyzed by a multifactor analysis of variance (multifactor ANOVA). The sensorial test was examined by a one-way ANOVA to compare samples according to sensory parameters. The LSD (least significant difference) procedure was used to test the differences between averages at the 5% significance level.

3. Results and Discussion

3.1. EOC-functionalized supports characterization

The silica microparticles functionalized with the antimicrobial compounds were characterized to confirm the immobilization of EOCs on the particles' surface. Table 1 shows the amount of antimicrobial compounds anchored to the supports and the surface charge of particles. Grafting was more efficient for vanillin as functionalized silica particles were obtained with the organic matter content anchored to the supports being 3-fold higher than for eugenol. The immobilization process changed the surface charge of the supports, as indicated by the Zeta potential results. The non-functionalized silica microparticles gave negative Zeta potential values due to the presence of silanol moieties on the supports' surfaces. In contrast, the supports functionalized with the bioactive compounds gave positive Zeta potential values because of the grafting of the EOC-alkoxysilane derivatives, which thus reaffirms the efficiency of functionalization.

Table 1. Content of the immobilized organic matter (α) and zeta potential values for the bare and functionalized silica microparticles.

SiO_2 support	**α (mg compound/g SiO_2)**	**Zeta potential (mV)**
Bare	-	-28.6 ± 3.2
Eugenol	35.8	30.9 ± 5.6
Vanillin	114.9	28.6 ± 1.0

3.2. Capability of the EOCs-functionalized supports used as filtering materials to cold-pasteurize apple juice

Figure 1 shows the *E. coli* K12 counts after the inoculation of the commercial apple juice, and also after filtering juice through a bed of silica particles (0.5 cm thickness, 2 g) (layer I) for both the bare support and for the support functionalized with either eugenol or vanillin. With vanillin, the experiment was also carried out with a bed of particles (1 cm thickness, 4 g) (layer II). As seen in Figure 1, filtering

juice through the non-functionalized support did not significantly reduce the inoculated bacterium (6.84±0.13 and 6.74±0.14 log CFU/mL for the unfiltered juice and the juice filtered through the control silica, respectively). In contrast, filtering apple juice through the EOC-functionalized supports significantly removed the *E. coli* load of the beverage ($p<0.05$). The use of the eugenol-functionalized support resulted in an approximate reduction of 5 logarithmic cycles. With vanillin, filtering juice through layer I of particles (0.5 cm thick) led to a 3-log reduction, which was not enough to reach the required pasteurization level. For this reason, the experiment was also carried out with a thicker bed of particles (1 cm; layer II), which resulted in a reduction of 5.5 log cycles. These results reveal that the removal capability of the EOC-functionalized particles' bed can be attributed to the interaction of the immobilized EOCs with the microbial cells to result in microbial retention. Free EOCs affect microbial cell membrane permeability, which leads to the leakage of cell contents and, in turn, results in irreversible damage and cell death (Burt, 2004). As recently reported, the immobilization of the EOCs on a support's surface allowed their antimicrobial properties to be preserved (Ruiz-Rico et al., 2017). After immobilization, the antimicrobial molecules at a high local concentration on the supports' surface could interact with microorganisms during the filtering process, which resulted in microbial damage. Besides, the efficiency of EOCs as preservatives in juice matrices has been previously demonstrated for eugenol (Ghosh, Mukherjee, & Chandrasekaran, 2014; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2008) and vanillin (Char, Guerrero, & Alzamora, 2009; Moon, Delaquis, Toivonen, & Stanich, 2006).

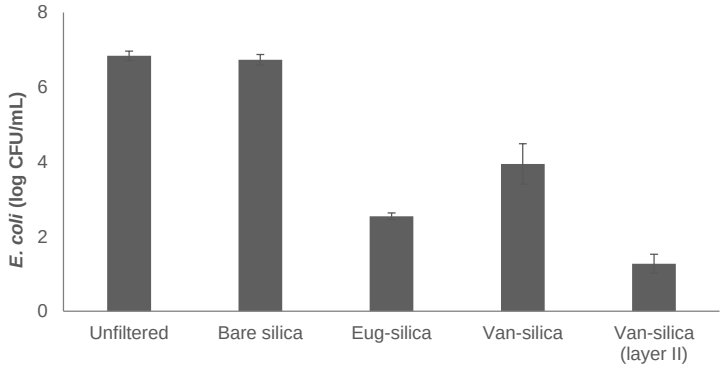

Figure 1. *E. coli* counts (log CFU/mL) in the inoculated apple juice before treatment and after filtering juice through a 0.5-cm bed of the bare silica microparticles and EOCs-functionalized supports. Layer II indicates a bed thickness of 1 cm. Mean value±SD (n=3).

Filtration through the EOC-functionalized supports played a significant role in the elimination of the *E. coli* inoculated in the apple juice samples (5-log reduction in the microbial count), which confirms the suitability of this system as cold pasteurization technology. This treatment allowed microorganisms to be removed at room temperature similarly to other emerging non-thermal technologies. Exposing the inoculated apple juice to pulsed light resulted in poor *E. coli* inactivation (ca. 3 log cycles), but this technology combined with ultrasound treatment led to an acceptable 5.9 log reduction (Ferrario & Guerrero, 2016). The inactivation of *E. coli* O157:H7 in the apple juice samples treated by pulsed electric fields resulted in a 4.5 log reduction in the work reported by Evrendilek et al. (2000). The combined use of ultraviolet radiation and pulsed electric fields gave a reduction of 6-7 log cycles, similarly to conventional heat treatment (Noci et al., 2008). High hydrostatic pressure has also provided promising results by achieving total *E. coli* inactivation (more than 5 log cycles) in fruit juice (Lavinas, Miguel, Lopes, & Valente Mesquita, 2008). Besides, a reduction of 7 log cycles in *E. coli* in apple juice has been achieved by dense phase carbon dioxide combined with mild heat treatment (Liao, Hu, Liao, Chen, & Wu, 2007).

3.3. Influence of filtration on the apple juice quality parameters during storage

After confirming the capability of the EOC-functionalized supports used as filtering elements to cold pasteurize apple juice, the effect of filtration on the microbiological and physic-chemical properties of apple juice during refrigerated storage was evaluated.

3.3.1. Microbiological parameters

Figure 2 shows the evolution of the microbial counts of mesophilic aerobic, psychrophilic and mold and yeast of the untreated and treated apple juices throughout the storage period. The results on day 0 indicated that the filtration process produced a microbial stabilization of the juice samples filtered through the EOCs-functionalized supports. It resulted in the total inhibition of native flora, whereas the unfiltered juice and the juice treated with the bare support had a microbial load of 1-2 log cycles of different microorganisms. These results agree with those obtained for the commercial apple juice inoculated with *E. coli* K12 (Section 3.2).

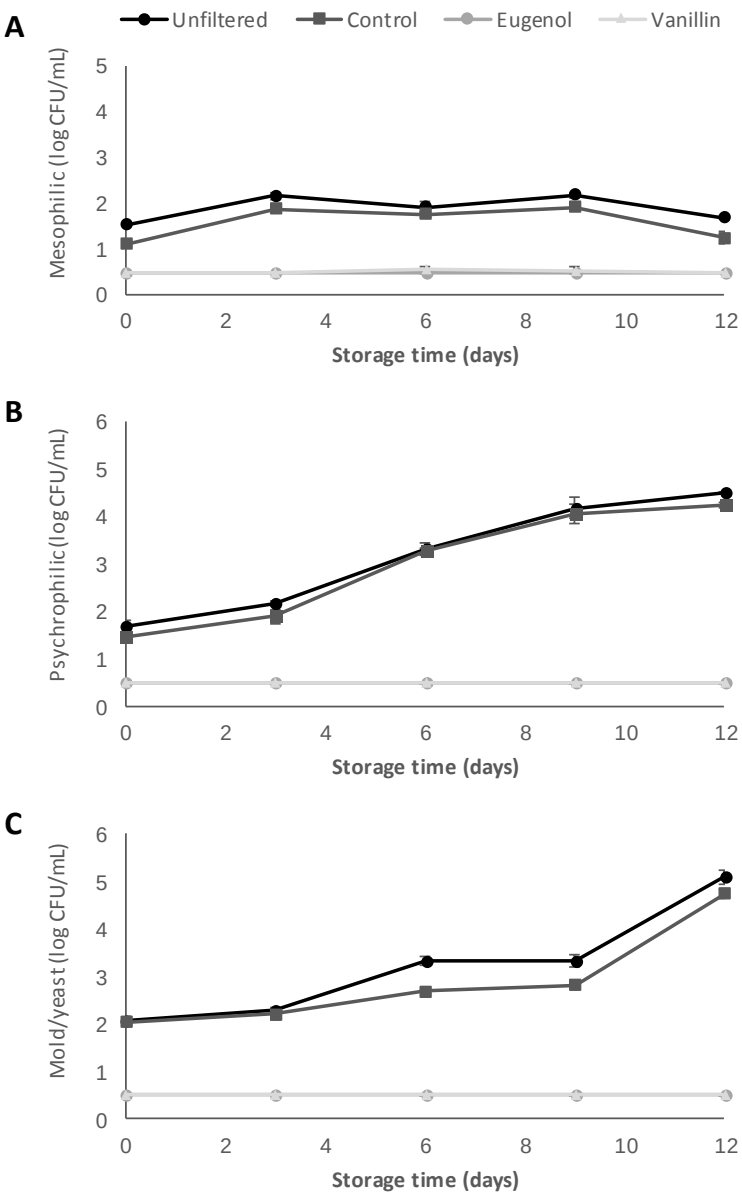

Figure 2. Microbial counts (log CFU/mL) of mesophilic aerobic (A), psychrophilic (B) and mold and yeast (C) of the unfiltered apple juice and the juice filtered through the non-functionalized particles (control) and EOC-functionalized supports during refrigerated storage. Mean values ±SD (n=3).

Regarding juice evolution during refrigerated storage, an increase in microbial counts was observed in response to storage time for both the unfiltered juice and the juice filtered through the non-functionalized supports. There was significant difference in the mean counts of the juice samples before and after filtering through bare particles ($p<0.05$). This partial microbial retention (less than 0.5 log cycles) endorses the very limited removal effect of the unmodified silica microparticles due to physical adsorption by weak forces on the filtering bed (Gialleli, Bekatorou, Kanellaki, Nigam, & Koutinas, 2016). In contrast, the juice samples filtered through the EOCs-functionalized supports led to the absence of microbiota throughout the 12 storage days. After 12 storage days, the unfiltered juice and that filtered with the unmodified support exceeded the acceptability limit of 100 CFU/g of the total counts established as a safety criterion for unpasteurized fruit and vegetable juices, according to Regulation (EC) No. 2073/2005 of the European Commission (European Union, 2005). Conversely, international guidelines recommend an acceptable level of aerobic mesophilic microorganisms values to be 10^4 CFU/g for pasteurized juices (Health Protection Agency, 2009).

Therefore, if we consider the product to be pasteurized juice, no sample exceeded the recommended limit after 12 storage days. In order to establish the shelf life of juices, the microbiological parameters were monitored until 120 days of apple juice storage at 4ºC. On day 30, the unfiltered juice and that filtered with the unmodified support gave a microbial count of ca. 4 log CFU/mL, which resulted in a shelf life of less than 30 days (Table 2). Conversely, the samples filtered through the EOC-functionalized supports sustained a total inhibition of native flora until day 120, when 1.4 log CFU/mL of mesophilic aerobic was enumerated for the juice treated with immobilized vanillin. Thus after filtering samples with the EOC-functionalized supports, the juices displayed microbial stability for more than 120 days, unlike the juices not treated with the immobilized bioactive compounds with a major increase in microbial load after 30 days.

These are remarkable results compared with other non-thermal treatments, such as microfiltration (Campos et al., 2002), pulsed electric field processing

(Evrendilek et al., 2000) and high hydrostatic pressure treatment (Lavinas et al., 2008), and provide a shelf life that lasts up to 60 days stored at 4°C. Moreover, the apple juice treatment done with pulsed light combined with ultrasound exceeded 4 log CFU/mL after 10 days of refrigerated storage (Ferrario & Guerrero, 2016). Consequently, the filtration using the EOCs-functionalized supports proves to be a preservation potential for developing 'fresh-like' apple juice with an equivalent or longer shelf life than thermal treatment in microbiological characteristics terms.

Table 2. Microbial counts (log CFU/mL) of mesophilic aerobic, psychrophilic and mold and yeast of the unfiltered apple juice and the juice filtered through the non-functionalized particles (control) and EOC-functionalized supports from day 30 to 120 of refrigerated storage. Mean values±SD (n=3).

Sample	Day	Mesophylic	Psychrophilic	Mould/yeast
Unfiltered	30	4.1 ± 0.0	5.2 ± 0.1	5.6 ± 0.1
	60	5.2 ± 0.2	6.5 ± 0.0	5.7 ± 0.2
	90	5.6 ± 0.1	6.3 ± 0.1	5.8 ± 0.2
	120	5.8 ± 0.3	6.5 ± 0.6	6.4 ± 0.6
Control	30	3.8 ± 0.1	5.3 ± 0.1	5.3 ± 0.3
	60	5.8 ± 0.0	6.6 ± 0.1	6.4 ± 0.0
	90	6.8 ± 0.2	6.9 ± 0.2	6.7 ± 0.3
	120	7.0 ± 0.2	7.3 ± 0.0	6.9 ± 0.1
Eugenol	30	nd	nd	nd
	60	nd	nd	nd
	90	nd	nd	nd
	120	nd	nd	nd
Vanillin	30	nd	nd	nd
	60	nd	nd	nd
	90	nd	nd	nd
	120	1.4 ± 1.2	1.6 ± 1.1	1.2 ± 1.1

nd: non detected

3.3.2. Physic-chemical parameters

Figure 3 shows the evolution of the physicochemical parameters (pH, acidity and °Brix) of the untreated and treated apple juice samples over a 12-day refrigerated storage period. After filtration (day 0), the unfiltered juice presented an average pH value of 3.6, and the juice filtered through the non-functionalized silica and eugenol-functionalized support obtained similar values (homogenous groups, $p<0.05$). In contrast, a significant difference was observed after filtering the apple juice through the vanillin-functionalized support, which had a higher pH value of 4.4. This value fell beyond the normal range of apple juice, which could lead to a different perception of taste and, consequently, to diminished consumer acceptability. This variation may be due to either the partial release of the immobilized bioactive compound (Table 3) or the presence of APTES moieties without reacting with the antimicrobial molecules on the supports' surfaces to provide the larger amount of alkoxysilane derivative immobilized on the support (Table 1). Regarding the evolution of the juice stored at 4°C, the pH values remained stable throughout the study period, and only slightly lowered during the storage of all the samples.

In acidity terms, a significant statistical difference was observed according to sample treatment ($p<0.05$), as shown in Fig. 3B. All the filtered samples presented significant differences with the untreated juice, but the acidity values obtained for the unfiltered juice and the juice filtered through the bare or eugenol-functionalized supports were similar. However, filtering juice through the vanillin-functionalized support modified this parameter more as acidity decreased. These results agree with the obtained pH values, as explained above.

Similarly to the acidity results, the processed juices significantly differed from fresh juice in soluble solid content terms ($p<0.05$), but these differences were minor and fell within the 4.7 and 4.8 °Brix range. In addition, the °Brix values of the different samples remained stable throughout the refrigerated storage.

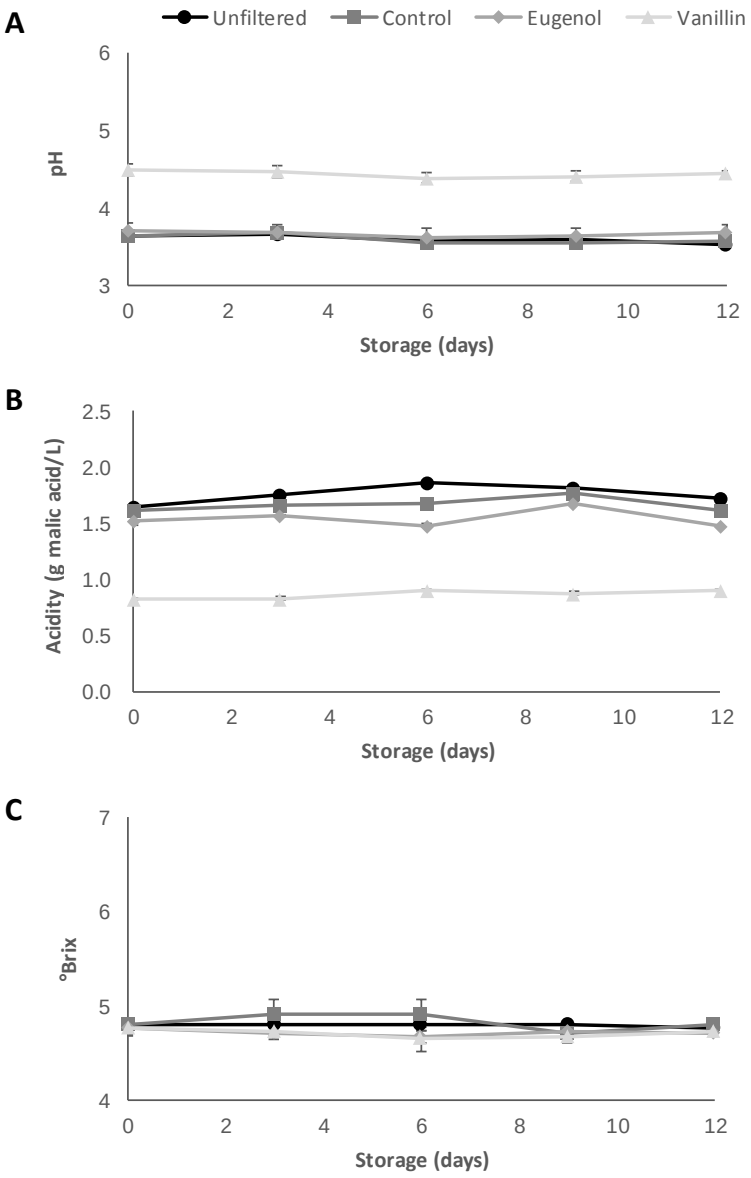

Figure 3. The pH, titratable acidity and °Brix values of the unfiltered fresh apple juice and after filtration through a bed of control silica support and the EOCs-functionalized supports during refrigerated storage. Mean values±SD (n=3).

The influence of the conventional preservation techniques on the physicochemical properties of fruit juices has been clearly stated, and justifies the need to develop mild preservation treatments. Thermal treatment has been reported to increase pH and is directly related with temperature, with pH values reaching ca. 4 under pasteurization conditions, which can entail a shorter shelf life because higher pH values could favor microbial growth (Charles-Rodríguez, Nevárez-Moorillón, Zhang, & Ortega-Rivas, 2007). Similarly, thermal pasteurization has been related with a significant decrease in acidity, which could be attributed to the degradation of organic acids with rising temperature (Aguilar-Rosas, Ballinas-Casarrubias, Nevarez-Moorillon, Martin-Belloso, & Ortega-Rivas, 2007).

In contrast, the treatment of fruit juices using filtration technologies did not significantly affect their physicochemical parameters, as studied in accordance with previous studies (Gialleli et al., 2016; Ortega-Rivas, Zárate-Rodríguez, & Barbosa-Cánovas, 1998). Similar results were achieved herein after filtering juice through the bare and eugenol-functionalized supports. The application of other non-thermal treatments, such as pulsed electric field, has been able to preserve pH, acidity and volatile compounds to a greater extent than thermal treatment (Aguilar-Rosas et al., 2007).

In addition, color was studied like the other physicochemical parameters defining juice quality. Figure 4 shows the evolution of L*, a* and b* of the apple juice samples during refrigerated storage. The statistical analysis revealed the significance of juice treatment and storage time on juice color evolution. In general, the juice filtered with the EOC-functionalized supports was brighter (higher L* values) than the unfiltered juice or that treated with the bare supports, which became darker (lower L* values). The a* values mostly differed for the juice treated with the non-functionalized supports, but with a significant increase (more red components), while the samples treated with the EOC-functionalized supports were slightly 'greener' (lower a* values) compared to the unfiltered samples. The b* values changed to a greater extent than the a* values, and resulted in more yellow components (higher b* values) for the treated samples than for the non-

filtered juice (control <vanillin <eugenol). The total color difference determination revealed the influence of the filtration process on the color of the samples. The ΔE value for the samples filtered through the non-functionalized support *versus* the non-filtered samples was 2.17. This color difference was at the same level as in previously reported studies into the thermal treatment of apple juice, in which heated juice obtained a ΔE of at least 2.69 compared to untreated juice (Krapfenbauer, Kinner, Gössinger, Schönlechner, & Berghofer, 2006). Filtering juice through the EOC-functionalized supports led to greater deviation, with ΔE values of 6.06 and 6.45 for the eugenol and the vanillin-functionalized supports, respectively.

Treating apple juice by ultrafiltration has led to significant browning in previous studies. Juice became darker according to the applied trans-membrane pressure, which could be attributed to enzymatic browning in relation to the difference in enzyme inactivation efficiency of techniques (Ortega-Rivas et al., 1998). In contrast, other non-thermal treatments, like pulsed electric field and ultraviolet radiation, have preserved apple juice color after treatment and throughout its shelf life, and has resulted in the chromatic coordinates being maintained (Evrendilek et al., 2000; Noci et al., 2008). Therefore, treating apple juice with the EOC-functionalized supports resulted in significant color differences, which is a weakness of this methodology compared to other techniques. Future studies should be conducted to assess how to overcome this limitation, probably by means of filtering many samples or with a previous washing with the food matrix.

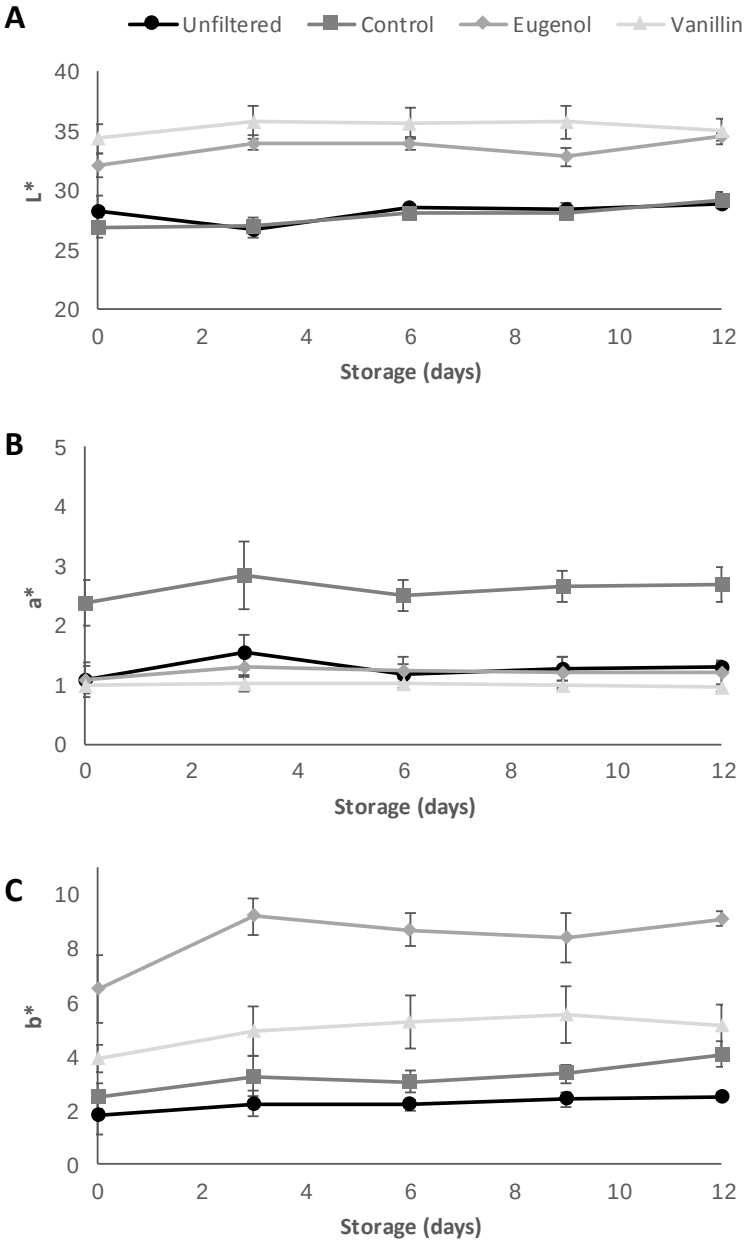

Figure 4. The CIEL*a*b* color coordinates of the unfiltered fresh apple juice and after filtering through a bed of control silica support and EOCs-functionalized supports during refrigerated storage. Mean values±SD (n=3).

3.4. Leaching the immobilized EOCs

The potential leaching of the grafted bioactive compounds was quantified to define the stability of the immobilization procedure and to determine the wash-out effects under the filtration conditions. Besides the amount of EOCs released from the supports, the percentage of leached compounds was calculated by considering the total EOCs attached to the supports (Section 3.1) and the amount of particles needed to prepare the bed of particles. Table 3 shows the amount of leached EOCs and the percentage of lixiviated compounds in juices after filtering through the EOC-functionalized supports. For immobilized eugenol, a zero wash-out effect was detected after filtering juice. In contrast, the juice filtered through the vanillin-functionalized support showed a 0.6% of the initial EOC's content grafted to particles' surface. Despite the low percentage of leached compounds, this partial release could be responsible for the impact of the treated juice on the physicochemical properties and could negatively affect sensory properties.

Table 3. EOCs leaching (mg) and the relative percentage of the leached EOC after filtering 100 mL of apple juice through the bed of the EOC-functionalized supports. Mean values±SD (n=3).

SiO_2 support	**Amount of released compound (mg)**	**Relative percentage (%)**
Eugenol	nd	nd
Vanillin	1.7 ± 1.3	0.6 ± 0.4

nd: non detected

Notwithstanding, it is important to highlight that covalent immobilization preserved the microbial stabilization potential of the EOC-functionalized supports. In addition, the low percentage of leached EOCs ratifies the safety and longevity of filtration technology because it would allow the possibility of a repeated or continuous reuse of immobilized compounds. However, the immobilization

procedure should be optimized to ensure the zero release of anchored molecules to prevent any influence on the physicochemical and sensory properties of juice.

3.5. Consumer acceptability of the filtered apple juice

According to the results of previous sections, the juices filtered through the most suitable support to stabilize their microbiota without leaching the immobilized molecule from the support were sensory-evaluated. Table 4 shows the sensory evaluation results of the juices filtered through the non-functionalized and eugenol-functionalized supports. As we can see, the juice treated with the eugenol-functionalized support was well accepted ($p<0.05$) great for the attributes evaluated by the sensory panel, except for odor.

Treating juice with conventional preservation methodologies (thermal pasteurization) causes sensory quality depletion and the appearance of odor and flavor defects (Manzocco, Plazzotta, Spilimbergo, & Nicoli, 2017). In contrast, the use of alternative non-thermal technologies, such as high-pressure carbon dioxide, pulsed electric field or high hydrostatic pressure, has resulted in the preservation of the sensory fresh-like features of treated juices (Kebede et al., 2018; Manzocco et al., 2017). Likewise, the apple juice subjected to filtration with the eugenol-functionalized support did not show any effect on the evaluated sensory attributes, which coincides with the results observed for the physicochemical parameters (Section 3.3.2).

Table 4. Scores of the different attributes evaluated in the apple juice filtered through the non-functionalized and eugenol-functionalized supports. Mean values±SD and median (n=44).

SiO_2 support	Appearance		Color		Odor		Acceptance	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
Control	5.8±1.5[a]	6	5.7±1.5[a]	6	6.7±1.5[a]	7	6.3±1.3[a]	6
Eugenol	7.2±1.0[b]	7	7.1±1.3[b]	7	6.9±1.5[a]	7	7.0±1.2[b]	7
α	*		*		ns		*	

Same letters in a column indicate homogeneous group membership. ns: non-significant, *p<0.05

The use of the silica support can be the equivalent to using clarification agents, such as bentonite, to eliminate the sediments and dark pigments that appear during apple juice processing, and to obtaining similar sensorial acceptability (Lauret, Sartori, Imaizumi, Brunelli, & Filho, 2018). Conversely, filtration methodologies that involve very small pore sizes, such as microfiltration and ultrafiltration, result in significant sensory changes with little color intensity and odor and flavor defects due to increase in the membrane retention of sugar, phenolic and other flavor components (Girard & Fukumoto, 1999).

Therefore, the sensory evaluation proved the suitability of the developed filtration treatment given the high attribute scores that confirm the preservation of the end product's major sensory attributes, as well as extending its shelf life beyond those obtained with conventional preservation methodologies.

4. Conclusions

In this study, silica microparticles functionalized with EOCs were applied as filtering materials for cold apple juice pasteurization. The filtration process, through the EOC-functionalized supports, allows to: (i) clarify juice to avoid turbidity and sediment in the end product; (ii) microbiologically stabilize the food matrix to increase its shelf life. In fact treating fresh juice with this technology eliminated its

native flora, and resulted in an apple juice with a much longer shelf life than that obtained by heat treatment.

The filtration process had a different influence on the physicochemical parameters of juice according to the immobilized bioactive molecule. The use of the eugenol-functionalized particles proved to be the more adequate support as it removed the product's microbial load without affecting its physicochemical parameters (pH, acidity, °Brix) or sensory profile, plus the zero release of the immobilized compound to the food matrix. Therefore, the proposed cold-pasteurization system has a high potential for treating fruit juices, as well as other liquid foods like milk, beer or wine.

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6. *GENERAL DISCUSSION*

The food industry faces huge challenges because of consumer trends. While there is increasing commitment to ensure food safety, awareness of the close relationship between nutrition and health exerts a great deal of pressure to provide 'natural' and 'fresh-like' products at affordable prices. In this context, the current doctoral thesis has been developed to design and apply a non-thermal technology for preservation of water and liquid food based on novel filtering aids.

Bearing in mind this objective, two materials were selected as filtering materials by their adsorption capabilities and functionalization features that allow the grafting of organic molecules. Despite the common nature of the chemical principles that both materials involve, the objectives and results were divided into two different chapters to offer a better understanding of the goals, methodology and relevant findings. Chapter 1 deals with the synthesis and the use as filtering systems of cellulosic materials obtained from a food by-product or functionalized with a bioactive organic molecule. Chapter 2 addresses the preparation and application of silica supports functionalized with natural antimicrobials as filtering materials. Both chapters were composed by different research articles. The results obtained in the different parts of the thesis that allowed the achievement of the general objective proposed in this work, have been discussed in detail in the five articles shown above. Hence this section aims to sequentially and relatedly discuss all the findings in order to address the issue holistically.

Filtration is a common technology used in the food and drink industry to concentrate, clarify and microbiologically stabilize food and food components. Filtration can be performed by membranes or microparticles from diverse materials, being the most relevant polymeric (cellulose or synthetic polymers) and siliceous (sand, bentonite, diatomite or silica) supports. Standard filtration using pore size between 1-1,000 μm allows only partial removal of microbial cells in beverages, that together with fouling problems and regeneration issues limit its application as non-thermal preservation technology. Thus the development of novel filtering materials were carried out with the aim to improve the features of the supports to microbial stabilize water and liquid foods, preserving the

organoleptic characteristics, increasing shelf-life, being easily applicable, environmentally acceptable and cost effective.

Chapter 1 focused on the development of cellulosic supports as filtering aids. In a first step, it was proposed to obtain and apply porous cellulosic particles from a corn by-product as an interesting cold pasteurization technology for the stabilization of water and orange juice using the particles in a continuous flow bioreactor (**Article 1**). Corn stalks were used by their lignocellulose composition with high porosity, also is eco-friendly material available in large quantities. A preliminary test with native corn stalks (NCS) and corn stalk tubular-cellulose (CSTC), showed effective removal of *S. cerevisiae* and *L. casei* from water. The microstructure of CSTC material, the variability on flow rate (0.3-2 L/day), the increment of the length of the filter (43 and 50 cm) and the initial microbial cell concentration (0.5-2 g/L) played a crucial role on the microbial load removal efficiency, as it was demonstrated in several filtration tests. The better conditions for the filtration system with CSTC were selected (50 cm height, *S. cerevisiae* concentration of 2 g/L, and flow of 1.5 L/day). Then, the continuous cold pasteurization of commercial water and orange juice was performed under the mentioned conditions to remove *S. cerevisiae* for 10 and 20 days respectively. The filter became clogged while the liquid passed through it in a continuous flow, so regeneration with hot water was necessary. Corn stalk-TC was a successful filtering material to retain yeast cells of contaminated commercial orange juice. However, the proposed treatment affected the color of the juice, which should be further studied to optimize the treatment in order to minimize the changes in this important sensory parameter.

Besides the development of new filtration materials to be used in the food industry, another relevant approach studied in this work was the functionalization of conventional filtering aids with bioactive molecules to improve the removal properties of existing materials. As proof of concept, commercial cellulosic paper was functionalized with polyamines to create cellulosic membranes capable of retaining the microbial load from a simple model matrix as drinking water (**Article**

2). With the immobilization of amino groups, a positive charged surface was obtained to retain bacteria negatively charged by attractive electrostatic forces. The covalent immobilization of a primary amine by means of the grafting of the organosilane *N*-(3-trimethoxysilylpropyl)diethylenetriamine on the surface of the paper allowed the filter to retain different concentrations of the Gram-negative microorganism *Escherichia coli* from water samples after a few seconds of contact with the filter. The covalent grafting of the amines on the cellulosic support could be reused without loss of efficiency and prevented the lixiviate of the immobilized molecules ensuring the safety of the treated water.

Therefore, the results obtained in Articles 1 and 2 (Chapter 1) confirmed the suitability of cellulosic supports, including the cellulose particles got from a food by-product or the commercial cellulose membranes functionalized with a bioactive molecule, to remove microorganisms from liquid foods with more advantages than conventional cellulosic filtering materials.

Given the potential in the microbial reduction of water observed for the filters made by the immobilization of a bioactive molecule on the surface of a filtering support, the next step in the thesis was the study of the covalent grafting of naturally-occurring antimicrobial compounds (carvacrol, eugenol, thymol and vanillin) on the surface of silica supports (Chapter 2). Silica particles were used as filtering support due to their stability and the easily modifiable-surface by the established organosilane chemistry that allows the incorporation of functional groups. Essential oil components were chosen as functional group because of their safety and their reported antimicrobial properties. The immobilization of essential oil components on the surface of silica particles of different mean size (5, 10, 25 or 50 µm) allowed the preparation of filtering materials able to microbiologically stabilize liquid foods. **Article 3** studied the application of the functionalized silica particles as filter aids for the disinfection of water. The removal capability of the supports depended on the particle size and the immobilized antimicrobial compound. In this sense, the biggest particles (50 µm) required a double layer to achieve the optimal retention, whereas particles of 10-25 µm displayed remarkable

retention properties. The results confirmed the bacterial removal capacity with log reduction values of 4 for an indicator of fecal contamination like *E. coli*, following the WHO requirements for household water treatment technologies. FESEM and fluorescence microscopy images showed that the antimicrobial activity of the functionalized supports was due to the combination of physical adsorption and the inactivation by contact with the immobilized EOCs. The interaction of EOCs with the microbial cell envelope results in disturbance of the cytoplasmic membrane and bacteria cell death, as a consequence of the electrostatic attraction between the negatively charged cell surface of the microorganisms and the positively charged EOC-functionalized supports. Finally, GC-MS analysis was used to study the potential leaching of the immobilized EOCs. No wash-out effects after filtration were observed, indicating a good stability of the EOCs bonding on the supports surface, with the exception of carvacrol-functionalized supports. The release of this compound from the silica surface could be due to an incorrect immobilization process or washing. Therefore, this work represents an important starting point for the development of new filtration procedures for ensuring water disinfection, preventing the disadvantages associated with conventional chemical disinfectants.

To assess the potential application of the silica supports functionalized with essential oil components in the beverage industry, **Article 4** evaluated these filtering systems in craft beer. The results showed the remarkable removal capability of the filtration technology against mesophilic bacteria, lactic acid bacteria, mold and yeast. The supports with mean size of 10 and 25 µm were the most effective filtering materials for reducing *E. coli*. Besides, the results demonstrated a significant influence of immobilized EOC on the removal properties. The microbial stabilization of the beer after filtration prevented the undesirable organoleptic changes as a consequence of the spoilage endogenous microflora. Thus, the cold pasteurization of craft beer could be performed by using the developed functionalized supports achieving the minimum requirements for safe and cold-pasteurized beer established by brewery experts. Besides, the sensory analysis of the treated beer showed minimal differences in the attributes

evaluated after filtration, which together with the zero release of the grafted molecules in the filtered beer, confirmed the great potential of the functionalized supports for the treatment of beverages.

Finally, the 50 µm-supports functionalized with eugenol and vanillin were selected to evaluate the removal capability of the developed filtering aids in the preservation of apple juice. **Article 5** studied the microbial stabilization of fresh apple juice by filtration with the functionalized supports and the influence of the filtration on its physico-chemical, microbiological and sensory properties throughout refrigerated storage. The suitability of the filtration as a cold pasteurization technology was firstly confirmed after obtaining at least a 5-log reduction of *E.coli* in commercial inoculated juice. The results using fresh apple juice showed the capability of the functionalized particles to eliminate its native flora, resulting in a much longer shelf life than conventional thermal treatment. The juice filtered through the vanillin-functionalized support showed a 0.6% of the initial EOC's content grafted to particles' surface on the food matrix, which affected the physicochemical and organoleptic properties of apple juice. On the other hand, filtering the juice with the eugenol-functionalized support had almost no effect on most of the physicochemical and sensory properties of the juice, which together with the no-wash out effect of the immobilized molecule, confirmed the potential of the filtration technology to provide microbiologically stable liquid food with acceptable quality.

Due to the high capacity of microbial retention of the supports developed in this thesis, it would be interesting to propose these supports as filtering elements in the beverage industry. Nevertheless, further studies are needed, to complete the optimization of these techniques. In this sense, some important parameters to be evaluated can be the filtration parameters (pressure, temperature, flow rate, etc.), the immobilization methodology to ensure zero release of the grafted compounds and the determination of the lifespan of the supports. Finally, it would be essential to carry out a complete sensory study of the final product and to determine the viability of the functionalized supports production in a pilot scale.

7. CONCLUSIONS

Conclusions

- Polymeric and silica filtering materials bare or functionalized with antimicrobial compounds have been successfully synthesize, characterize and apply in filtration of different liquid foods.
- The delignified corn stalk-tubular cellulose material has been effectively developed and applied as filtering material for water and orange juice. The system working in continuous flow has been able to retain microorganisms. However, the treatment affects the color of the juice, which is an important disadvantage of this technique. Further studies should be carry out to avoid this problem.
- Polyamines-functionalized cellulose membranes have been applied as filtering materials capable to remove pathogenic bacteria in water, which offer new alternatives for *in situ* water treatment.
- Silica supports with different mean particle size have been functionalized with essential oil components for their use as filtering materials able to remove both pathogenic and spoilage microorganisms of the beverages, with non-significant effect on the physicochemical and organoleptic properties of the food matrix, and even extending the shelf life of the product.
- Zero or very low release of immobilized essential oil compounds to the food matrix have been obtained after filtering through the functionalized supports confirming the covalent immobilization of the molecules on the supports' surface.
- The proof-of-concept studies of this thesis demonstrate the potential incorporation of the proposed filtering technology in the food industry. To achieve the transfer of this technology, it is necessary to conduct previous pilot-tests to ensure the future implementation on an industry scale.

8. *APPENDICES*

Appendix I. Abbreviations and Acronyms

Ag	Silver
ANOVA	Analysis of variance
APTES	(3-Aminopropyl)triethoxysilane
BET	Brunauer, Emmett and Teller model
BJH	Barret, Joyner and Halenda model
CECT	Colección Española de Cultivos Tipo
CFU	Colony-forming unit
CSTC	Corn Stalk Tubular Cellulose
DBPs	Disinfection by-products
DE	Diatomaceous earth
DPCD	Dense phase carbon dioxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSMZ	German Resource Centre for Biological Material
EFSA	European Food Safety Authority
EOs	Essential oils
EOCs	Essential oil components
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FESEM	Field Emission Scanning Electron Microscopy
FT-IR	Fourier transform infrared spectroscopy
GAC	Granular activate carbon
GC-MS	Gas chromatography–mass spectrometry
GRAS	Generally Recognized As Safe
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HPP	High pressure processing
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
log	Logarithm
LAB	Lactic acid bacteria
LRV	Log reduction value
LSD	Least significant difference
MF	Microfiltration

MgO	Magnesium oxide
$MgSO_4$	Magnesium sulfate
N3	*N*-(3-trimethoxysilylpropyl)diethylenetriamine
NaCl	Sodium chloride
NaOH	Sodium hydroxide
N_2	Nitrogen
NF	Nanofiltration
NC	Nanocellulose
NCS	Native Corn Stalks
NH_4SO_4	Ammonium sulfate
PBS	Phosphate Buffer Solution
PCA	Plate count agar
PEF	Pulsed electric fields
PL	Pulsed light
RSF	Rapid sand filtration
SEM	Scanning electron microscope
SiO_2	Silicon dioxide
SSF	Slow sand filtration
SP	Silica particles
TBX	Tryptone bile x-glucuronide agar
TC	Tubular cellulose
TCM	Tubular cellulose material
TGA	Thermogravimetric analysis
TiO_2	Titanium dioxide
TSB	Tryptic soy broth
UF	Ultrafiltration
US	Ultrasound
UV	Ultraviolet
WHO	World Health Organization

Appendix II. Predoctoral stage at a foreign institution

Food Biotechnology Group, Department of Chemistry, University of Patras, Greece. From 5 September 2018 to 5 December 2018, under the supervision of Athanasios Koutinas.

Appendix III. Other scientific contributions

Oral presentation:

N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Liquid food pasteurization by filtration through particles functionalized with natural antimicrobial compounds*. 4th International & 5th National Student Congress of Food Science and Technology. Valencia, February of 2018.

Posters:

N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Effect of filtration through particles functionalized with antimicrobial compounds on the quality and shelf life of apple juice.* 5th International & 6th National Student Congress of Food Science and Technology. Valencia, February of 2019.

N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Influence of free and encapsulated essential oils on shelf life of fish and meat burgers.* 5th International & 6th National Student Congress of Food Science and Technology. Valencia, February of 2019.

J. Mazo, N. Aullé, N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Beer sterilization by filtration through amorphous silica particles functionalised with bioactive natural compounds*. XI International Workshop on Sensors and Molecular Recognition. Valencia, July of 2017.

N. Aullé, J. Mazo, N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Desarrollo de un sistema de esterilización de agua mediante filtración a través de partículas de sílice amorfa funcionalizadas con compuestos activos de aceites esenciales.* XI International Workshop on Sensors and Molecular Recognition. Valencia, July of 2017.

N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Development of paper membranes functionalized with bioactive compounds for the reduction of Escherichia coli in drinking water.* XI International Workshop on Sensors and Molecular Recognition. Valencia, July of 2017.

N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Development of filters functionalized with polyamines for the reduction of Escherichia coli in drinking water.* IV National and III International Student Congress of Food Science and Technology, Valencia, February of 2017.

N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat. *Study of inhibitory activity of antimicrobial filters against Enteroccocus hirae.* IV National and III International Student Congress of Food Science and Technology, Valencia, February of 2017.