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Additional Information

Enhanced antimicrobial activity of essential oil components immobilized on silica particles

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Abstract

The antimicrobial activity of essential oils components (EOCs) is well-known. However, their high volatility and powerful aroma limit their application in the formulation of a wide range of food products. In this context, the antimicrobial activity of carvacrol, eugenol, thymol and vanillin grafted onto the surface of three silica supports with different morphologies, textural properties and chemical reactivities (fumed silica, amorphous silica and MCM-41) was evaluated herein. Materials characterization revealed a good immobilization yield and all the devices showed a micro-scale particle size. Sensory evaluation revealed that sensory perception of EOCs decreases after covalent immobilization. Moreover, immobilization greatly enhanced the antimicrobial activity of the essential oil components against *Listeria innocua* and *Escherichia coli* compared to free components. The incorporation of EOCs immobilized on silica particles into pasteurized milk inoculated with *L. innocua* demonstrated their effectiveness not only for *in vitro* conditions, but also in a real food system.

Keywords: carvacrol; eugenol; thymol; vanillin; immobilization; silica support; *Listeria innocua*; milk.

1. Introduction

New techniques to prevent food spoilage and to guarantee food safety have rapidly and innovatively developed in recent years as a result of the current inadequacy of traditional antimicrobial methods and the growing spread of antibiotic resistant strains of bacteria and fungi (Capeletti et al., 2014). Some new tendencies in this field include the use of naturally-occurring antimicrobial compounds, e.g., plant metabolites. Essential oils (EOs), lipophilic extracts of bioactive compounds with antimicrobial activity against several food-borne microorganisms have grown the most in research publications and industrial applications (Hyldgaard, Mygind & Meyer, 2012).

The antimicrobial activity of EOs has been attributed to their phenolic compounds and their interaction with microbial cell membranes, which cause the leakage of ions and cytoplasmic content, and can thus lead to cellular breakdown (Burt, 2004). Despite the described antimicrobial behavior, the direct application of EOs to food products has several limitations: strong sensory properties (Nostro & Papalia, 2012), high volatility (Majeed et al., 2015), poor water solubility (Burt, 2004), degradability (Turek & Stintzing, 2013) and potential toxicity (Smith et al., 2005). Moreover, the concentration of an essential oil component (EOC) needed to inhibit microbial growth in a food system is higher than in *in vitro* studies. This is not due only to interactions with food matrix components (Hyldgaard et al., 2012), but also to difficulties in their dispersion in the food water phase (Weiss, Gaysinsky, Davidson & McClements, 2009).

Hence research has focused on the development of technologies, such as encapsulation or immobilization, to improve the functionality of natural antimicrobials (Weiss et al., 2009).

Regarding encapsulation, different organic delivery systems have been used to encapsulate EOs for their later application directly or after incorporation to films or coatings for food preservation purposes (Guarda, Rubilar, Miltz & Galotto, 2011; Ravichandran, Hettiarachchy, Ganesh, Ricke & Singh, 2011; Ribes, Fuentes, Talens & Barat, 2016).

Besides traditional organic matrices, new inorganic materials (i.e. porous siliceous materials) have been used as supports to prepare antimicrobial devices through the encapsulation of a payload molecule in the voids of porous silica particles. Of all of them, MCM-41-like supports

are the most widely used porous silica in applications in the food sector, where they can be used as catalysts in the synthesis of nutrients and bioactive molecules, in sensor technology and also as carriers to design smart delivery systems (Pérez-Esteve, Ruiz-Rico, Martínez-Máñez & Barat, 2015). Besides porous materials, other silica particles are widely used in animal feed and in the food industry (Uboldi, Giudetti, Broggi, Gilliland, Ponti & Rossi, 2012). This is the case of amorphous silica, particles which are considered GRAS, an authorized additive in Europe and E-551-classified (Contado, Ravani & Passarella, 2013).

Entrapment of antimicrobial compounds in these materials can also protect bioactive substances from environmental stress, mask undesirable sensory properties, prevent interactions with food components, and achieve the controlled release of the antimicrobial compound at the site of action. Bearing in mind these features, different naturally-occurring antimicrobial compounds, such as allyl isothiocyanate (Park, Barton & Pendleton, 2012), caprylic acid (Ruiz-Rico et al., 2015), lysozyme (Yu et al., 2015), and EOCs (Bernardos et al., 2015), have been encapsulated in mesoporous silica supports. It is noteworthy that all these studies have managed to preserve or enhance the inhibitory effect of bioactive compounds.

Apart from voids capable of entrapping active compounds, siliceous materials present a large surface capable of reacting with organic molecules, and of creating hybrid organic-inorganic systems where silica materials act as a support and organic molecules create a functional layer on the support's surface. Based on this approach, Li & Wang (2013) reported lysozyme-coated mesoporous silica nanoparticles that exhibited efficient enhanced antibacterial activity against *Escherichia coli*. Qi, Li, Yu and Wang (2013) used vancomycin-modified mesoporous silica nanoparticles to kill pathogenic gram-positive bacteria. Pędziwiatr-Werbicka et al. (2014) synthesized fatty acids functionalized mesoporous silica particles with relative antimicrobial activity. Despite these promising results, the preparation of antimicrobial devices from EOs by this innovative approach has not yet been explored as far as we know.

Accordingly, this study aimed to design a collection of antimicrobial devices based on anchoring several volatile EOCs (carvacrol, eugenol, thymol and vanillin) to the surface of three types of silica particles with different surface areas, textural properties and chemical reactivities

(silica-fumed, MCM-41 and amorphous silica), and to evaluate their antimicrobial activity against some food-borne pathogens, e.g., *Listeria innocua* and *Escherichia coli*, compared with that of free bioactive compounds.

2. Materials and methods

2.1. Chemicals

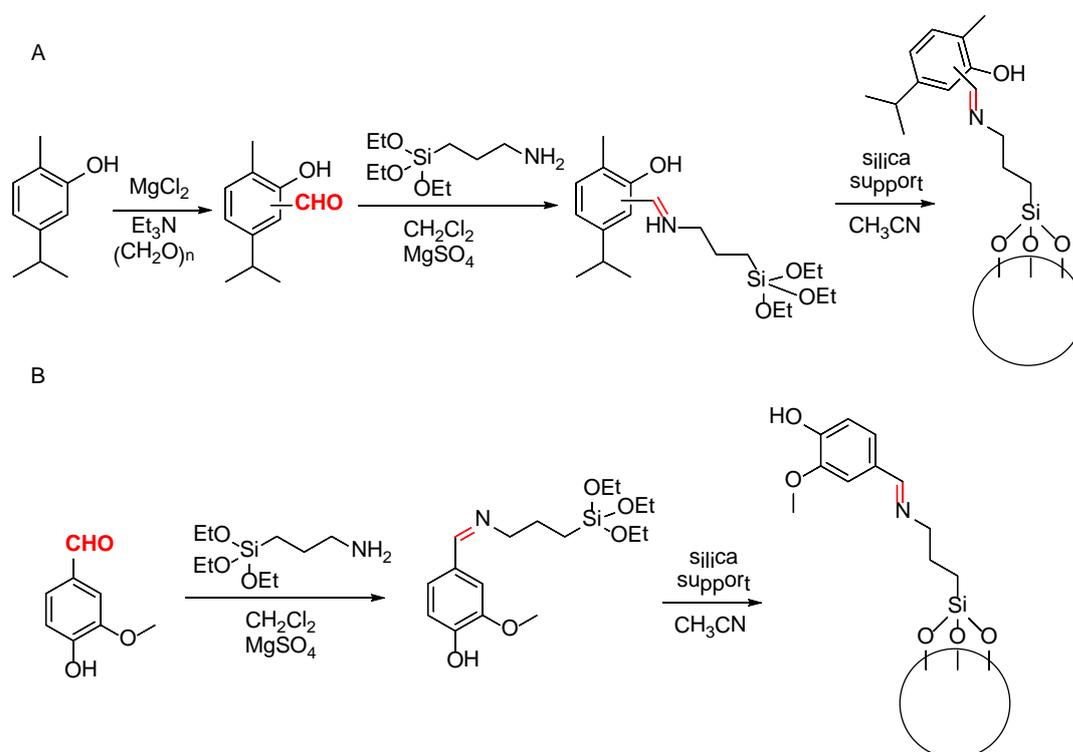
N-cetyltrimethylammonium bromide (CTABr), sodium hydroxide, triethanolamine (TEAH₃), tetraethylorthosilicate (TEOS), (3-Aminopropyl)triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butanone, dimethyl sulfoxide, carvacrol, eugenol and thymol were provided by Sigma-Aldrich (Madrid, Spain). Vanillin was purchased from Ventós (Barcelona, Spain). Acetonitrile, hydrochloric acid, magnesium sulfate, potassium hydroxide, sulfuric acid and microbiological media grade were provided by Scharlab (Barcelona, Spain). Fumed silica (FS) nanoparticles (AEROSIL® 200) were purchased from Evonik Industries (Essen, Germany) and amorphous silica (AS) microparticles (SYLYSIA® SY350/FCP) were provided by Silysiamont (Milano, Italy).

2.2. Mesoporous silica particles synthesis

MCM-41 microparticles were synthesized following the so-called “atran route”, where CTABr was used as the structure-directing agent. The molar ratio of the reagents was fixed at 7 TEAH₃: 2 TEOS:0.52 CTABr:0.5 NaOH:180 H₂O. CTABr was added to a TEAH₃ and NaOH solution, which contained TEOS at 118 °C. After dissolving CTABr in the solution, water was slowly added with vigorous stirring at 70 °C to form a white suspension. This mixture was aged at 100 °C for 24 h. Following synthesis, the solid was recovered, washed with deionized water and dried at 70 °C. The as-synthesized microparticles were calcined at 550 °C in an oxidant atmosphere for 5 h to remove the template phase (Ruiz-Rico et al., 2016).

2.3. Preparing the antimicrobial devices

A three-step synthetic procedure was followed to prepare the antimicrobial devices (see Scheme 1). In the first step, carvacrol, eugenol and thymol were transformed into aldehyde derivatives. In a second step, the aldehydes of carvacrol, eugenol and thymol and pure vanillin were reacted with 3-aminopropyltriethoxysilane (APTES) to yield the corresponding alkoxy silane derivatives. Further anchorage of the four silane derivatives to the external surface of three different inorganic scaffolds (FS, MCM-41 and AS particles; third step) resulted in the preparation of the final 12 solids.



Scheme 1. Representation of the synthesis procedure of the antimicrobial devices. A. The three-step synthesis of the carvacrol, eugenol and thymol immobilized systems (carvacrol provided as an example). Step 1. Aldehyde derivatization. Step 2. Alkoxy silane derivatization. Step 3. Anchoring to the silica support (FS, MCM-41 or AS). B. The two-step synthesis of the vanillin immobilized systems. Step 1. Alkoxy silane derivatization. Step 2. Anchoring to the silica support (FS, MCM-41 or AS).

2.3.1. Preparing the EOC aldehyde derivatives

The antimicrobial activity of terpenoids (carvacrol and thymol) and phenylpropanes (eugenol) is linked to the kind and number of substituents on the aromatic ring, and it has been shown that the hydroxyl group is important for antibacterial activity (Hyltdgaard, Mygind & Meyer, 2012). Therefore, the aldehyde derivatives of carvacrol, eugenol and thymol were synthesized for the purpose of adding a second reactive moiety capable of reacting with the amine group of alkoxy silane, and of maintaining hydroxyl group free of the EOCs whose presence is critical for antibacterial activity (Gill & Holley, 2006). Presence of an own aldehyde group in the vanillin structure avoided this step.

The carvacrol and thymol aldehydes were synthesized by direct formylation using paraformaldehyde. In a typical synthesis, 150 mL of acetonitrile, 40 mmol of carvacrol or thymol, 150 mmol of trimethylamine and 40 mmol of anhydrous $MgSO_4$ were placed inside a round-bottomed flask. The mixture was placed in an argon atmosphere and stirred for 15 min at room temperature. Afterward, 270 mmol of paraformaldehyde were added and the reaction mixture was refluxed for 3.5 h at 83 °C. The mixture was then allowed to cool at room temperature. The solution was acidified with 5% HCl solution (320 mL) and stirred for 30 min in an inert atmosphere. Finally, the organic portion was extracted with diethyl ether and volatiles were removed under reduced pressure to obtain the carvacrol or thymol aldehyde.

Eugenol aldehyde was synthesized using a Reimer–Tiemann reaction. In a typical synthesis, 150 mL of water were heated at 80 °C in a round-bottomed flask, and 22 mmol of eugenol were dissolved in the water. When the temperature had fallen to 60 °C, 400 mmol of KOH and 88 mmol of chloroform were added. As the reaction was exothermic, chloroform was added at a rate of 1 mL/h over a 7-hour period for safety reasons. The reaction mixture was kept at 60 °C for a further 8-hour period. Then the solution was acidified with 10 % H_2SO_4 solution. The organic portion was extracted with n-butanone and volatiles were removed under reduced pressure.

2.3.2. *Preparing the EOC-alkoxysilane derivatives*

To facilitate the covalent anchoring of the four EOCs to silica supports, the corresponding EOCs-alkoxysilane derivatives were synthesized. For this purpose, 2 mL of the carvacrol, thymol or eugenol and unmodified vanillin aldehydes were reacted with 2.3 mL (10 mmol) of (3-aminopropyl)triethoxysilane (APTES) in the presence of dichloromethane (20 mL) and MgSO₄. The mixture was stirred in reflux for 1 h to be then filtered and evaporated under reduced pressure to give a transparent liquid.

2.3.3. Synthesis of the EOC-functionalized silica particles

In a typical synthesis, 1 g of bare particles (FS, MCM-41 or AS) was suspended in 40 mL of acetonitrile in a round-bottomed flask in an inert atmosphere. Then the excess alkoxy silane derivative was added and the final mixture was stirred for 5.5 h at room temperature. Finally, solids were filtered off, washed with acetonitrile and distilled water, and dried at room temperature in vacuum for 12 h.

2.4. Materials Characterization

Bare and functionalized silica supports were characterized by standard techniques: morphological analysis, particle size distribution, zeta potential and determination of the degree of functionalization. A morphological analysis was performed by field emission scanning electron microscopy (FESEM) observations. FESEM images were acquired by a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. Particle size distribution was determined by a Malvern Mastersizer 2000 (Malvern Instruments, UK). To take measurements, solids were dispersed in tryptic soy broth (TSB). All the measurements were taken in triplicate on previously sonicated highly dilute dispersions. To determine the zeta potential, a Zetasizer Nano ZS (Malvern Instruments, UK) was used. Solids were dispersed in TSB at the 1 mg/mL concentration and were sonicated for 2 min to preclude aggregation. The zeta potential was calculated from the particle mobility values by applying the Smoluchowski model. The degree of functionalization of the different particles was determined by thermogravimetric analyses (TGA) and elemental analysis. TGA determinations were made

on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc., Schwarzenbach, Switzerland), with a heating program that consisted in a heating ramp of 10 °C per minute from 273 to 373 K, followed by an isothermal heating step at this temperature for 60 min in a nitrogen atmosphere (80 mL/min). Then the program was allowed to continue with a dynamic heating segment from 373 to 1273 K in an oxidant atmosphere (air, 80 mL/min) and with an isothermal heating step at this temperature for 30 min. The bulk density of the different silica supports was determined by pouring around 20 g of support into a 100-mL measuring cylinder and tapping 10 times on a flat wooden platform. The volume occupied by the sample was recorded. The mass of the empty and filled measuring cylinders, and the final volume occupied by each sample, was noted. Bulk density was expressed as the mass/volume ratio (g/cm³). The data acquired from the different characterization techniques were used to calculate the number of particles/g of solid, EOC content/g of particle and the EOC density on the particles' surface. The number of particles per gram of solid was calculated according to Eq. 1. The content of the EOCs (determined by the TGA and elemental analysis) and the mean average particle size (determined by laser diffraction) values were used to estimate the number of EOC molecules/g solid (Eq. 2). The surface area was taken into account to calculate the density of EOCs on the particles' surface (Eq. 3).

$$\text{Number of particles/g solid} = 1 / ((\text{particle's volume (cm}^3) \times \text{density (g/cm}^3)) \quad (\text{Eq. 1})$$

$$\text{EOC molecules/g solid} = (\alpha_{\text{EOC}} (\text{g/g solid}) / \text{molecular weight (g/mol)}) \times 6.023 \times 10^{23} \quad (\text{Eq. 2})$$

$$\text{EOC density (molec/nm}^2) = \text{EOC molecules (molec/g solid)} / \text{surface area (nm}^2/\text{g solid)} \quad (\text{Eq. 3})$$

2.5. Microbiological Assays

Strains *L. innocua* (CECT 910) and *E. coli* K12 (CECT 433) were obtained from the Colección Española de Cultivos Tipo (CECT; Valencia, Spain). Bacterial stocks were stored at 4 °C in plate count agar (PCA) before use. The cells from a colony of *L. innocua* or *E. coli* grown on PCA were transferred to 10 mL of TSB and were incubated at 37 °C for 24 h to obtain an inoculum with a density of approximately 1 x 10⁸ cells/mL of broth.

2.5.1. Antimicrobial susceptibility assays

The antimicrobial activity of the EOCs was determined by the macrodilution method. Different ranges of concentrations were tested for each EOC according to bibliographical data (Belda-Galbis, Leufven, Martínez & Rodrigo, 2014; Burt, 2004; Cava-Roda, Taboada-Rodríguez, Valverde-Franco & Marín-Iniesta, 2012; Guarda et al., 2011). Equivalent amounts of the immobilized EOCs were calculated according to the results obtained when characterizing the degree of functionalization (*vide infra*). The EOC stock solution in dimethyl sulfoxide (DMSO) or the particles stock suspension in TSB (functionalized FS, MCM-41 and AS particles) was prepared. To achieve the final concentrations of the free or immobilized bioactive compounds, different volumes of stock suspensions were added to 15 mL of TSB in Erlenmeyer flasks. Then flasks were inoculated with 5 or 50 μL of inoculum for *E. coli* and *L. innocua*, respectively, to provide an initial cell density of approximately 10^5 CFU/mL, and were incubated with orbital stirring (150 rpm) at 37 °C for 24 h. All the treatments were set in triplicate. Positive and negative controls were included in all the assays.

After incubation, viable cell numbers were determined as colony-forming units (CFU) by the spread plate technique using selective media (Palcam agar supplemented with polymyxin B, acriflavine and ceftazidime for *L. innocua*; Tryptone Bile X-glucuronide (TBX) agar for *E. coli*) and were incubated at 37 °C for 24 h (*E. coli*) or 48 h (*L. innocua*). These values were logarithmically transformed and expressed as \log_{10} CFU/mL.

2.5.2. Antimicrobial effect of EOCs on a real food system

The capability of the free and MCM-41 immobilized thymol and vanillin to control *L. innocua* growth in pasteurized skimmed milk was evaluated by simulating 7-day refrigeration storage. Equivalent concentrations of the free and immobilized EOCs (0.05, 0.25 and 0.5 mg/mL for thymol; 0.5, 0.75 and 1 mg/mL for vanillin) were added to 15 mL of sterilized milk and were then inoculated with 10^2 CFU/mL of the microorganism. Samples were stored with stirring at 4

°C for 7 days. On days 0, 1, 3, 5, and 7, samples were taken and counted by the spread plate technique (see Section 2.5.1. for details).

2.6. Sensory evaluation

A sensory evaluation was conducted to assess the capability of the immobilization process to mask the odor impact of the EOCs in skimmed milk. Thymol was chosen as reference due to its strong smell and its enhancement of antimicrobial activity after the immobilization on the silica supports. A selected and trained profiled panel of 12 assessors performed the sensory evaluation. The members of the panel were selected and trained according to the guidelines of the International Standard Organization (ISO 8586, 2012).

Assessment was carried out following the guidelines for the use of quantitative response scales to obtain a quantitative assessment of the intensity of perception of the thymol typical aroma (ISO 4121, 2003). Assessors were trained in preliminary sessions to identify thymol typical aroma which were calibrated using aqueous solutions of the EOC at different concentrations (0, 0.05, 0.1, 0.2, 0.3 and 0.5 mg/mL). Panelists rated the aroma intensity using a structured numerical scale of 5-point (1=not perceptible, 2=barely intense, 3=moderately intense, 4=very intense, 5=extremely intense). Thus, the panel evaluated thymol intensity aroma on skimmed milk fortified with free or MCM-41 immobilized thymol. The amounts of free and immobilized thymol added to the milk were selected according to the assays in the real food matrix (see Section 2.5).

2.7. Statistical analysis

Data were statistically processed using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). The influence of different supports and concentrations of treatments on bacterial viability was analyzed by an analysis of variance (multifactor ANOVA). The LSD (least significant difference) procedure was used to test differences between averages at the 5% significance level.

Multiple regression analyses with the stepwise removal procedure were performed to study the relationship between different variables (EOC type, support type, EOC concentration, particle concentration, number of particles, mean size, zeta potential, EOC content and EOC density on particles' surface) and the dependent variable (bacterial growth) in the antimicrobial susceptibility assays. For the analysis, EOC type and support type were introduced as dummy variables.

3. Results and discussion

3.1. Designing the antimicrobial devices

Four EOCs were used in this study: carvacrol and thymol (major components of *Lamiaceae* family plants), eugenol (a major component of clove and cinnamon oil), and vanillin (a primary component of vanilla bean extracts). They all were selected for their recognized antimicrobial properties (Burt, 2004; Hyldgaard et al., 2012), them being a “generally recognized as safe” (GRAS) component, and for their restricted use in certain food industry applications as a result of limited water solubility and/or intense spicy/medicinal/sweet aroma, which justifies anchoring to mask their sensory properties (Shah, Davidson & Zhong, 2012).

Three different silica particles were selected as the inorganic support: AS, MCM-41 and FS. Amorphous silica particles are non-crystalline structures of silicon dioxide produced in different sizes. MCM-41 is a mesoporous material with a hierarchical structure developed firstly by Mobil Oil Corporation researchers with high stability, large specific surface area and volume, controllable size, easy surface functionalization and high biocompatibility (Aznar, Oroval, Pascual, Murguía, Martínez-Máñez & Sancenón, 2016). Finally, fumed silica are microscopic droplets of amorphous silica with a branched chainlike primary structure that consists of fused SiO₂ nanoparticles that agglomerate in three-dimensional secondary and tertiary particles (Walls et al., 2000). FS particles have an extremely low bulk density, a large surface area and, above all, high chemical reactivity.

As detailed in the experimental section, carvacrol, eugenol and thymol were first chemically modified to include an aldehyde moiety. The efficiency of the formylation process was

evaluated by the ^1H NMR analysis. For all the EOCs, the product yield estimated from the ^1H NMR spectra was 20-40%. Then the aldehyde-containing carvacrol, eugenol and thymol derivatives and vanillin (which already contain an aldehyde group) were reacted with APTES. The resulting mixture was then reacted with FS, MCM-41 and AS to obtain a total of 12 EOC-functionalized silica particles.

3.2. Characterizing the EOC-functionalized silica particles

The morphological characterization of the bare and EOC-functionalized silica particles by field emission scanning electron microscopy (FESEM) is shown in Figure 1. For materials characterization, the silica particles functionalized with thymol were chosen as reference supports. Bare FS appears as an irregular-shaped sponge-like structure, probably formed by the aggregation of primary particles (reported particle size of 12 nm). Bare MCM-41 particles are seen as dense microparticles with a clear hexagonal morphology and a mean single-particle size of ca. 4 μm . Finally, AS is shown as being sphere-like with a rough morphology, similar to fumed silica supports. In all cases, the appearance of particles did not change after the functionalization process.

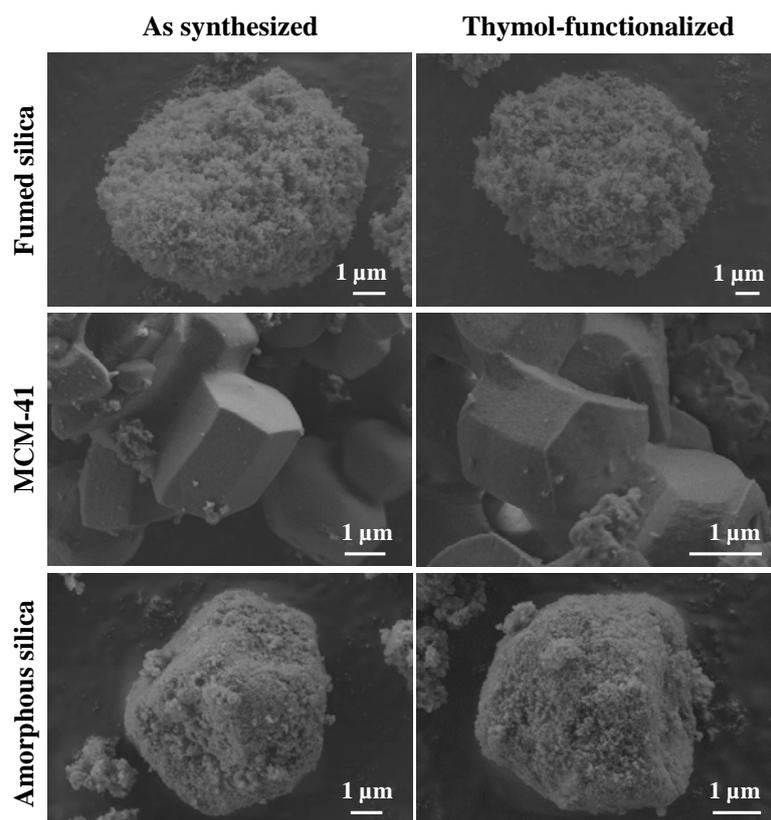


Figure 1. Characterization of particle size and particle shape by the FESEM of the bare and thymol-functionalized fumed silica, MCM-41 and amorphous silica materials.

The particle size distribution of the bare and functionalized materials in the presence of the culture broth is shown in Table S1. The bare FS particles showed a mean size distribution within the nanoscale range, which suggests the disaggregation of the sponge-like structures observed by FESEM. After the functionalization, particle size remained on the microscale, which suggests that the formation of an organic layer on the surface of particles stabilizes the original clusters. The bare MCM-41 particles displayed a similar size distribution to that determined by the FESEM. The immobilization of EOCs on the MCM-41 surface increased the mean particle size. Finally, the bare AS materials showed a similar size distribution on the microscale. After functionalization, size heterogeneously increased.

Aggregation tendency can be explained by their zeta-potential values (Table S1), which fell within the instability range (-30 mV to +30 mV) so that the interaction between particles and some culture broth components were expected to form aggregation clusters (Ruiz-Rico et al.,

2015). These data suggest that all the functionalized particles presented a microscale size range. Hence these supports were unable to be endocytosed by bacteria and the whole antimicrobial effect was due to the bacteria that came into contact with the particle's surface.

The contents of EOCs (carvacrol, eugenol, thymol and vanillin) attached to the different solids (FS, MCM-41 and AS) were determined by elemental and thermogravimetric analyses (Table 1). These values were used to calculate the amount of the different solids required to evaluate the equivalent concentrations of the free and immobilized EOCs.

Table 1. Content (α) in grams of EOCs per gram of SiO₂ for the solids of fumed silica (FS), mesoporous silica (MCM-41) and amorphous silica (AS) particles.

	$\alpha_{\text{carvacrol}}$ (g/g SiO ₂)	α_{eugenol} (g/g SiO ₂)	α_{thymol} (g/g SiO ₂)	α_{vanillin} (g/g SiO ₂)
FS	0.0084	0.0499	0.0072	0.0886
MCM-41	0.0181	0.0652	0.0758	0.1886
AS	0.0368	0.0585	0.0149	0.0838

3.3. Antimicrobial activity of the EOC-functionalized silica particles against *L. innocua*

The antimicrobial activity of the free and developed immobilized EOCs was established by determining the bacterial growth of two food-borne microorganisms by representing an example of gram-positive (*L. innocua*) and gram-negative bacteria (*E. coli*). Different ranges of concentrations were tested for each EOC in line with the literature (Belda-Galbis et al., 2014; Burt, 2004; Cava-Roda et al., 2012; Guarda et al., 2011). Figure 2 shows the bacterial count of *L. innocua* after 24 h of treatment with free and immobilized carvacrol, eugenol, thymol and vanillin. The inoculated initial microbial density is shown as a dotted black line in the graphs, and allows us to distinguish if presence of the free or immobilized EOCs at different concentrations triggered a bacteriostatic or bactericide effect, or not, after 24 h of incubation.

For carvacrol (Figure 2A), addition of the free molecule to the culture broth within the 0.001-0.025 mg/mL range provoked very low antimicrobial activity (ca. 2-5% of reduced growth). However, the immobilization of EOCs on the three silica supports led to a reduction of between

2-6 logarithmic cycles of *L. innocua* at the same concentrations. At higher concentrations, the antimicrobial effect of the free carvacrol increased with the amount of added EOC, and reached total microbial growth inhibition at concentrations that equaled or were higher than 0.15 mg/mL. These results agree with previous studies, which have reported that treatment with 0.2 mg/mL of carvacrol brought about a reduction of 3 logarithmic cycles of *L. monocytogenes* after 5 h of treatment (Ait-Ouazzou, Espina, Gelaw, Lamo-Castellví, Pagán & García-Gonzalo, 2012). After immobilization, the minimum bactericidal concentration (MBC) was halved (0.075 mg/mL) when carvacrol was immobilized on the FS support, which demonstrates the positive effect of immobilization at both low and high concentrations.

Eugenol (Fig. 2B) affected *L. innocua* growth at higher concentrations than carvacrol; 1 mg/mL of free eugenol reduced the microorganism to undetectable levels, which is in accordance with the study of Gill and Holley (2006). The EOC displayed significantly greater antimicrobial activity after immobilization on MCM-41 and FS supports at low concentrations. Indeed, concentrations over 0.025 mg/mL and 0.3 mg/mL for the eugenol immobilized on the FS and MCM-41 supports, respectively, had a bactericidal effect. Despite this improvement, the MBC value remained at the same level. The eugenol-immobilized AS support progressively reduced the growth rate and had a bacteriostatic effect rather than bactericidal activity.

Thymol immobilization was the most remarkable enhancement of the antimicrobial effect. While addition of the free molecule at concentrations between 0.01 and 0.075 mg/mL only provoked reduced growth at about 1-3%, addition of immobilized thymol increased the antimicrobial effect, with a bactericidal effect that fell within the 0.004-0.012 mg/mL range for the different supports. In fact MBC was established at 0.05 mg/mL for the thymol-functionalized FS and 0.075 mg/L for the thymol immobilized on the MCM-41 and AS particles. Addition of the free molecule did not reach the MBC within the whole range of concentrations studied in accordance with the results reported by Xiao, Davidson and Zhong (2011), who stated that 0.02 mg/mL of thymol was able to reduce, but not inhibit, the microbial growth of *Listeria monocytogenes* in a growth medium. It should be taken into account that although thymol and carvacrol are isomers, the bactericidal effect of the two free compounds

differed. While free carvacrol exhibited an MBC of 0.15 mg/mL, the MBC was not reached by free thymol, not even at concentrations of 0.2 mg/mL. After immobilization, the antimicrobial of both isomers was similar (an MBC of 0.05-0.075 mg/mL), which implies a more marked improvement of the antimicrobial activity of thymol.

Finally, vanillin had to be added at high concentrations to have an antimicrobial effect like that indicated in Figure 2D and in previous studies (Cava-Roda et al. 2012; Fitzgerald, Stratford, Gasson, Ueckert, Bos & Narbad, 2004). In fact at free vanillin concentration of 2 mg/mL, only a reduction of 4.5 logarithmic cycles was achieved. Despite the poor antimicrobial activity of the free molecule, immobilization of vanillin on silica supports improved its antimicrobial activity on all the supports. Complete inhibition of *L. innocua* was accomplished after the treatment with 2 mg/mL of vanillin immobilized on the MCM-41 particles.

The enhancement of the antimicrobial activity of immobilized bioactive compounds in silica particles has been previously reported in few articles. Botequim et al. (2012) developed mesoporous silica particles coated with a quaternary ammonium cationic surfactant such as didodecyldimethylammonium bromide (DDAB) and studied their antimicrobial activity against bacteria, yeast, moulds, and viruses. Results showed that coated mesoporous silica particles had a lower minimum inhibitory concentration than that obtained for free DDAB. In a later study, Li and Wang (2013) reported lysozyme-coated mesoporous silica particles as antibacterial agents with 5-fold higher antibacterial activity against *E. coli* than that of free lysozyme. This enhanced antibacterial efficacy originated from the lysozyme corona favoured by the multivalent interaction between the immobilized lysozyme and peptidoglycans of the bacterial walls, which produced the hydrolysis of peptidoglycans and damaged the bacterial cell wall. More recently, Vico et al. (2016) designed fumed silica particles functionalized with a naturally-occurring antioxidant and antimicrobial compound such as gallic acid. Immobilized gallic acid displayed enhanced stability and increased antimicrobial activity against *Paenibacillus larvae*. The inhibitory effect of functionalized fumed silica particles was between 14 and 27 times higher than the free polyphenol. The improvement of antimicrobial activity may be because the

transport of the polyphenol into the cell is favoured by the nanoparticles, which allow larger local concentrations of gallic acid, thus enhancing membrane disruption mechanisms.

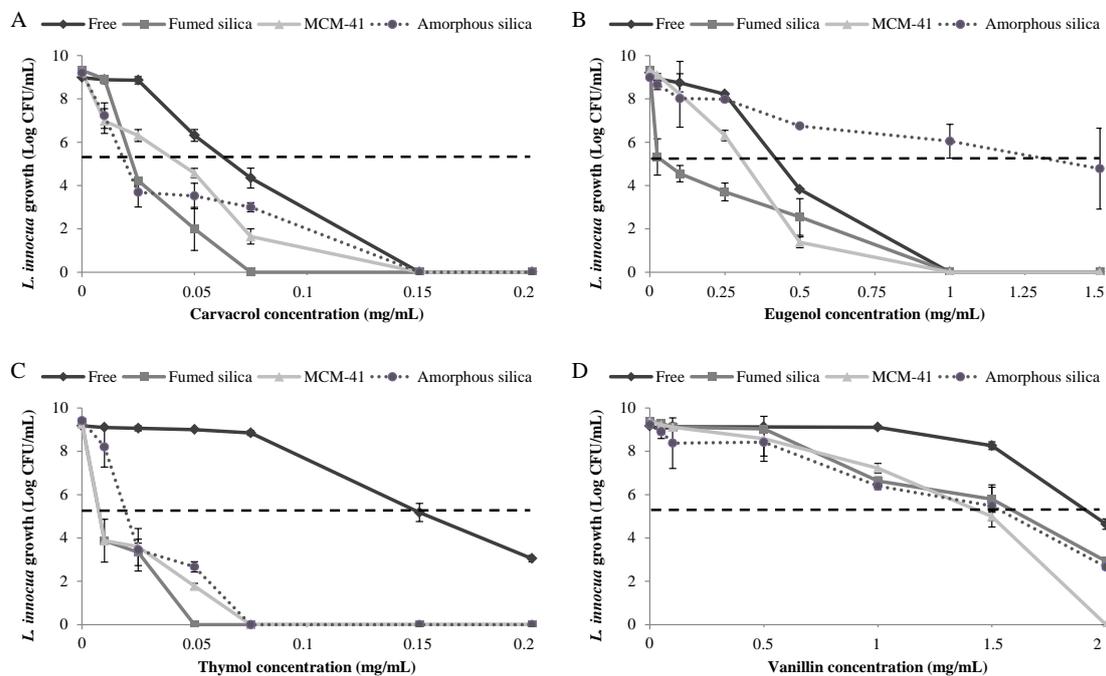


Figure 2. Growth (log CFU/mL) of *L. innocua* after incubation with free and immobilized carvacrol (A), eugenol (B), thymol (C) and vanillin (D) according to the EOC concentration (means and standard deviations, n=3).

After demonstrating the effect of the immobilization of EOCs on reducing bacterial growth, a multiple regression analysis was performed to study the influence of the independent variables (EOC type, support type, EOC concentration, particle concentration, number of particles, mean size, zeta potential, EOC content and EOC density on the particles' surface) on the bacterial count. Table S2 shows the multiple linear regression model for the bacterial count for each EOC used. The R-Squared statistics indicates that the model fit explained 91.57% of variability in the bacterial count. As seen in the table, the statistical analysis confirmed the significant effect of EOC type, particle concentration, zeta potential and EOC density on bacterial growth, and how thymol was the most effective bioactive compound. Other independent variables did not appear to have any significant effect on microorganism inhibition, despite them being related to some

significant independent variables (i.e. EOC density depended on EOC content and mean particle size which, in turn, depended on zeta potential and type of solid). According to these data, the amount of particles and the EOC anchored to particles significantly determined the studied support's antimicrobial activity.

3.4. Antimicrobial activity of the EOC-functionalized silica particles against *E. coli*

Following the same procedure for *L. innocua*, the antimicrobial activity of the four free and immobilized EOCs included herein was tested against *E. coli*. As reported by other authors, *E. coli* was resistant to the treatment with carvacrol and vanillin at high concentrations of 0.2 mg/mL and 2 mg/mL, respectively (Ait-Ouazzou et al., 2012; Guarda et al., 2011; Cava-Roda et al., 2012). Conversely to *L. innocua*, in which the poor effectiveness of these two free molecules significantly improved after immobilization on silica supports, immobilization did not lead to significant antimicrobial improvements against this bacterium (data not shown).

Conversely, strong *E. coli* growth inhibition took place after adding the immobilized eugenol and thymol to the growth broth. Figure 3A shows the bacterial count of *E. coli* after incubation with the free and immobilized eugenol. As observed, addition of the free molecule at concentrations between 0.025 and 0.25 mg/mL only reduced the growth rate. In contrast, the treatment with 0.25 mg/mL of eugenol immobilized on FS and MCM-41 had a bactericidal effect with bacterial counts of 3.64 ± 0.40 and $4.45 \pm 0.01 \log_{10}$ CFU/mL for the eugenol-functionalized FS particles and MCM-41 particles, respectively. The MBC of free eugenol was established as 0.5 mg/mL in accordance with other studies (Dušan, Marián, Katarína & Dobroslava, 2006), whereas a concentration within the range of 1-1.5 mg/mL of immobilized EOC was needed to achieve total microorganism inhibition, except for the AS support.

The antimicrobial effect of thymol against *E. coli* is shown in Figure 3B. Neither free nor immobilized thymol caused any growth inhibition at the 0.01-0.05 mg/mL concentrations. However, high concentrations (0.075-0.2 mg/mL) of immobilized thymol on the MCM-41 and AS supports displayed improved antimicrobial activity compared to the free bioactive compound. So whereas the use of free thymol did not cause 100% growth inhibition at any of

the tested concentrations, an MBC of 0.15 and 0.2 mg/mL was found for the immobilized thymol in the MCM-41 and AS particles, respectively.

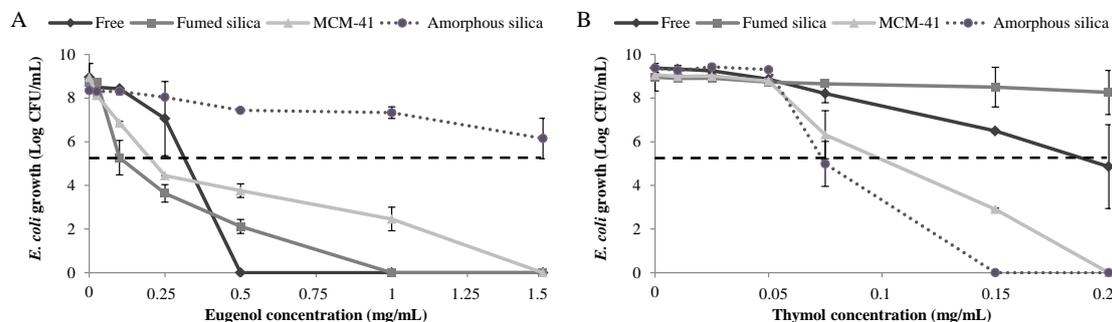


Figure 3. Growth (log CFU/mL) of *E. coli* after incubation with the free and immobilized eugenol (A) and thymol (B) according to EOC concentration (means and standard deviations, n=3).

It has been generally established that gram-positive bacteria are more sensitive than gram-negative ones when different EOCs are used (Ait-Ouazzou et al., 2012; Burt, 2004). This behavior is in agreement with the results obtained herein. The poorer sensitivity of gram-negative bacteria to the action of the different EOCs could be due to differences in the structure and permeability of the bacterial cell membrane, such as the outer membrane that surrounds gram-negative microorganisms restricting the diffusion of hydrophobic compounds (Guarda et al., 2011).

According to several authors, the mechanism of action of EOCs on bacteria cells is based on the alteration of the cellular envelope of microorganisms. EOCs disrupt the cellular membrane with morphology modifications and cell permeabilization, cause pH homeostasis alterations, leakage of inorganic ions and loss of membrane potential, and inhibit cellular respiration and perturb lipid fractions of bacterial cytoplasmic membranes (Ait-Ouazzou et al., 2013; Fitzgerald et al., 2004; Gill & Holley, 2006). The simultaneous presence of a free hydroxyl group, a delocalized electron system in the molecule and a hydrophobic character that allows membrane

accumulation appear essential for the antimicrobial activity of these EOCs (Nostro & Papalia, 2012).

A multiple regression analysis was also performed to study the influence of the independent variables (EOC type, support type, EOC concentration, particle concentration, number of particles, mean size, zeta potential, EOC content and EOC density on particles' surface) on *E. coli* growth. Table S3 shows the multiple linear regression model for the two EOC ($R^2 = 96.86\%$). In this case, the significant independent variables were EOC type, EOC support, mean size, zeta potential and number of particles. The significant variables differed from the *L. innocua* results, but the statistical results were consistent between microorganisms; i.e., particle concentration, zeta potential and mean size determine number of particles. The support type also had a significant influence for the eugenol-functionalized solids, where FS particles were the most effective. The regression model indicated that the smaller the particle size, the lower the bacterial count. A large number of particles in suspension enhanced the antimicrobial effect because the probability of a particle coming into contact with bacterial cells was higher.

3.5 Antimicrobial effect of EOCs on a real food system

Finally, in order to assess the antimicrobial activity of the developed supports against *L. innocua* in real food, the antimicrobial activity of two solids that exhibited the greatest antibacterial activity was tested in pasteurized skimmed milk. Drinking pasteurized milk has been associated with some outbreaks of listeriosis due to recontamination after heat treatment (Cava-Roda et al., 2012). *Listeria* is able to grow under refrigeration and survive in freezing environments (Gandhi & Chikindas, 2007). Therefore, the combined use of antimicrobials with refrigerated storage can be a suitable approach to prevent pathogen bacteria from proliferating in milk (Belda-Galbis et al., 2014).

Figure 4 shows the microbial growth of *L. innocua* inoculated in pasteurized skimmed milk (control) and milk after incorporating the free and MCM-41 immobilized thymol and vanillin. Presence of a lower free thymol concentration (0.05 mg/mL) in inoculated milk did not appear to have a significant inhibitory effect compared to the positive control. The ineffectiveness of

this thymol level agrees with previous studies, such as Xiao et al. (2011), which showed no effect after 0.04 mg/mL of thymol treatment on reduced fat milk. Higher concentrations displayed bacteriostatic activity over time, which improved with increasing EOC concentrations. Similar results were obtained for the treatment with the thymol-functionalized solid, particularly for the highest concentration (0.5 mg/mL). The antimicrobial activity of vanillin (Fig. 4B) was slower than the *in vitro* results due to the incubation conditions (4 °C) and the food matrix composition. Nevertheless, immobilized vanillin displayed better antimicrobial activity than the free compound, which allowed microorganisms to grow. Low immobilized-vanillin concentrations had a bacteriostatic effect, whereas the highest concentration (1 mg/mL) reduced microbial growth by 1.5 logarithmic cycles on incubation day 3.

The differences to the *in vitro* results were caused mainly by refrigerated storage and the interaction with some food matrix components. The literature establishes that bactericidal concentrations increase for lower temperatures, probably due to the activation of the stress response in hostile environments. Microorganisms modify their membrane composition to increase their cold tolerance, so EOC resistance may also increase (Belda-Galbis et al., 2014). EOC can also interact with hydrophobic constituents (lipids and proteins) of complex food systems, such as milk, with diminished antimicrobial effectiveness (Cava-Roda et al., 2012; Shah et al., 2012).

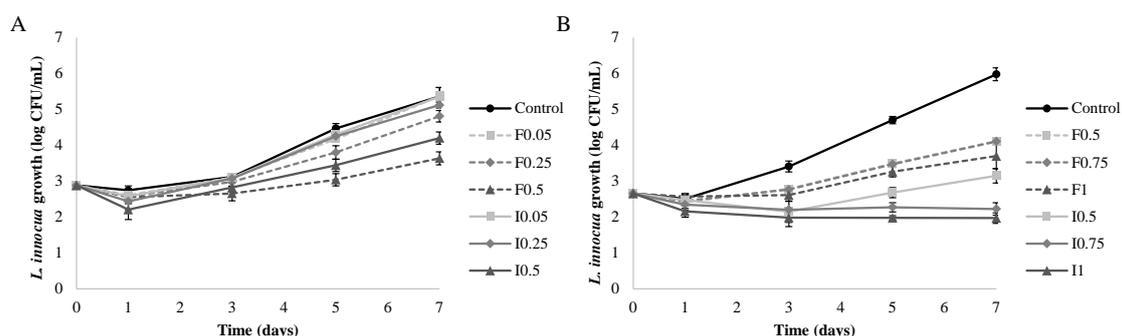


Figure 4. Growth (log CFU/mL) of *L. innocua* in skimmed milk with the free and immobilized thymol (A) and vanillin (B) during 7 storage days at refrigeration temperatures (means and standard deviations, n=3).

3.6. Sensory evaluation

To evaluate the effect of the immobilization to reduce the impact of EOC incorporation on the aroma of food products, a sensory evaluation was performed by a trained panel. Bearing in mind the responses of the intensity of thymol perception into milk fortified with free and immobilized thymol, it was estimated the percentage of reduction of the odor perception after the immobilization of the EOC. The results obtained indicated a significantly reduction of $88.5\pm 1.7\%$ of the aroma intensity of the immobilized thymol incorporated to pasteurized skimmed milk. These results confirm that feasibility of the immobilization to avoid the impact of the EOC on the sensory profile of a real food matrix.

4. Conclusions

We report herein the synthesis, characterization and evaluation of a collection of 12 antimicrobial devices based on anchoring carvacrol, eugenol, thymol and vanillin to the surface of silica supports of different particle sizes, and with distinct textural properties and chemical reactivities. Preserving the antimicrobial activity of EOCs allowed us to conclude that the proposed immobilization methodology allows the functional hydroxyl moiety of carvacrol, eugenol, thymol and vanillin to be preserved. Despite the fact that different EOC-silica support combinations yielded distinct antimicrobial activity, the antimicrobial effect against *L. innocua* and *E. coli* of all the EOCs-functionalized supports improves compared to free compounds. These results suggest that the immobilization of EOCs onto silica supports might be considered a novel strategy to develop a new generation of antimicrobial systems that may not only enhance the antimicrobial activity of EOs, but also mask their characteristic odor/taste.

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