Eugenol and thymol immobilised on mesoporous silica-based material as an innovative antifungal system: application in strawberry jam

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Abstract

Essential oils and their main compounds have been studied in-depth for their antifungal properties against a wide variety of microorganisms. However, the strong odour emitted by them, even at low concentrations, makes their incorporation into food matrices difficult. Immobilisation of antimicrobial compounds on solid surfaces could be a strategy to reduce their odour impact. The antifungal effectiveness of eugenol and thymol bioactive agents, free and immobilised on mesoporous silica microparticles (MCM-41 family), and their impact on the final aroma and fungal decay of strawberry jam, were evaluated herein. Free eugenol and thymol exhibited good antifungal properties against the fungi strains tested, and thymol proved more effective. The antifungal activity of immobilised eugenol and thymol displayed greater antifungal activity for immobilised eugenol. The jams prepared with immobilised eugenol on MCM-41 microparticles exhibited no mould and yeast development during the studied storage time. The sensory evaluation confirmed that eugenol and thymol immobilisation reduced their typical strong impact on strawberry jam flavour. This work demonstrates the promising use of immobilised eugenol on mesoporous silica microparticles to control strawberry jam decay.

Keywords: Antifungal activity; eugenol; thymol; mesoporous silica support; strawberry jam.
1. Introduction

Yeast and moulds can grow on raw and processed foods where the environmental conditions for most bacteria are unfavourable (Krisch, Tseremmadmid, & Vágvölgyi, 2011). These microorganisms are broadly distributed and able to decay different food commodities, such as wine, cheese, vinegar, juices, fruits, sugar and meat (Gammariello, Conte, Lucera, Mastromatteo, & Del Nobile, 2014). Chemical preservatives have been extensively used in recent years to control fungi development. Nevertheless, their negative consumer perception and changes in national regulations have forced food manufacturers to produce food commodities free of chemical additives (Ribes, Fuentes, Talens, & Barat, 2016).

Essential oils (EOs) are natural volatile compounds from aromatic plants with a strong odour (Burt, 2004). Many EOs have been generally recognised as safe (GRAS) by the Food and Drug Administration (FDA) in 21 Code of Federal Regulations 182.20 (CFR, 2015). Their high content in phenolic derivatives, e.g., eugenol and thymol, etc. (Abbaszadeh, Sharifzadeh, Shokri, Khosravi, & Abbaszadeh, 2014; Zabka & Pavela, 2013), make the antifungal properties of EOs a good alternative to synthetic chemical preservatives. Eugenol is a naturally-occurring phenol extracted from buds and leaves of clove (Ribes et al., 2016) that is effective against fungi due to cytoplasmic membrane disturbance (Mihai & Popa, 2015). Thymol is the main monoterpane phenol found in the EOs extracted from Lamiaceae family plants, with strong antifungal activity against a wide range of fungal microorganisms, including Aspergillus and Penicillium species, among others (Klarić, Kosalec, Mastelić, Piecková, & Pepeljnak, 2006). However, the concentration of both compounds required to control fungal decay in foods modifies the food product’s sensory profile given their strong flavour. For this reason, research that
seeks for alternatives to minimise the sensorial impact of EOs on food products that do not diminish their antimicrobial effectiveness are very important. A potential approach is the immobilisation bioactive compounds from EOs on surfaces.

Among the potential supports to immobilise active molecules, siliceous materials like mesoporous silica particles are very promising thanks to their unique features, such as stability, biocompatibility and large load capacity (Bernardos & Kourimska, 2013). In this context, MCM-41 (Mobil Composition of Matter) is a member of the mesoporous materials’ family according to the IUPAC (International Union of Pure and Applied Chemistry) classification (Dünder-Tekkaya & Yürüm, 2016), known to have a large specific surface and specific volume, and is easy to functionalise and highly stable. MCM-41-based materials have also been reported to resist harsh conditions of the stomach, acid matrices and microbial action (Pérez-Esteve, Ruiz-Rico, Martínez-Máñez, & Barat, 2015b). The chemical formula for MCM-41 is SiO₂, which is a common additive (E551) in the food industry (Barahona, Ojea-Jimenez, Geiss, Gilliland, & Barrero-Moreno, 2016). Given their easy preparation and properties, MCM-41 particles have been used in the present work as promising silica supports where bioactive agents can be immobilised.

The main purpose of this work was to investigate the feasibility of immobilised eugenol and thymol on mesoporous silica particles (MCM-41 family) as an antifungal system, and to study their antifungal effectiveness and sensory impact of the materials on strawberry jam.
2. Materials and methods

2.1 Microbial strains, culture media and chemicals

Strains Aspergillus flavus (CECT 20156), Aspergillus niger (CECT 20156), Penicillium expansum (CECT 20140), Zygosaccharomyces rouxii (CECT 1229) and Zygosaccharomyces bailii (CECT 12001) were used as test microorganisms, and were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). For mould species, potato dextrose agar (PDA) and potato dextrose broth (PDB) were used, while yeast peptone dextrose broth (YPDB) and agar were employed for the yeast species. All the culture media were purchased from Scharlab (Barcelona, Spain).

Eugenol (99% w/w) and thymol (≥ 99% w/w) were provided by Sigma-Aldrich (Madrid, Spain). For the synthesis of MCM-41 microparticles and the derivatisation and immobilisation of the antifungal compounds, N-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), triethanolamine (TEAH₃), tetraethylorthosilicate (TEOS), (3-Aminopropyl)triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butaneone, dimethyl sulfoxide (provide all of them by Sigma-Aldrich, Madrid, Spain), acetonitrile, hydrochloric acid (HCl), magnesium sulphate (MgSO₄), potassium hydroxide (KOH) and sulfuric acid (H₂SO₄) (Scharlab, Barcelona, Spain) were employed.

2.2 Antifungal activity of free eugenol and thymol

Free bioactive compounds eugenol and thymol were individually examined against A. flavus, A. niger and P. expansum, as described by Manso, Cacho-Nerin, Becerril, and Nerín (2013) with minor modifications. Spore suspensions of 10⁶ CFU/mL were prepared in NaCl (0.7% w/v) and Tween 80 (0.1% w/v), and confirmed using a
hematocytometer. MIC (Minimal Inhibitory Concentration) values were obtained by macrodilution in Erlenmeyer flasks that contained 15 mL of PDB and 1% (w/v) of Tween 80 to secure the total active compounds dispersions. A solution of 1,000 mg/kg of thymol was obtained by dissolving the appropriate amount in dimethyl sulfoxide. Different concentrations of bioactive compounds were tested: 0.1, 0.2, 0.3 and 0.4 mg/mL. The control samples, with no antifungal agents, were prepared following the same procedure. Each Erlenmeyer flask that contained free eugenol and thymol were inoculated with 100 µL of the spore suspension and incubated under orbital stirring (180 rpm) at 25 °C for 72 h. The results were expressed as log CFU/mL.

After incubation, the lowest eugenol and thymol non-growth concentration was established as the MIC. To determine the minimal fungicidal concentration (MFC), 100 µL of the non-growth suspensions were seeded onto Petri plates prepared with 15 g of PDA. MFC was defined as the lowest concentration at which no colonies developed after 72 h of incubation at 25 °C.

The antifungal effectiveness of free eugenol and thymol against Z. rouxii and Z. bailii was also evaluated, for which the methodology followed was similar to that described in the mould assays. Cell suspensions of 10^6 CFU/mL were prepared in Tween 80 (0.1 % w/v), and confirmed using a hematocytometer. The MIC values were obtained by macrodilution in Erlenmeyer flasks that contained 15 mL of YPDB and 1% (w/v) of Tween 80 to secure the total bioactive compounds dispersions. The preparation of the thymol solution and the control samples, and the concentration of the tested bioactive agents, were the same as those previously described. Each Erlenmeyer flask that contained free eugenol and thymol was inoculated with 100 µL of 10^6 CFU/mL,
and incubated under orbital stirring (180 rpm) for 48 h at 25 °C. The results were expressed as log CFU/mL.

After incubation, the MIC and the MFC values were determined as described above for moulds, but by employing YPD agar as the culture media. All the tests were conducted in triplicate.

2.3 Study of mesoporous silica particles

2.3.1 Synthesis of MCM-41 microparticles

Synthesis of the mesoporous MCM-41 microparticles was carried out using the so-called “atrane route” described by Pérez-Esteve et al. (2015a). The molar ratio of the reagents was fixed at 7 TEAH$_3$: 2 TEOS:0.52 CTABr:0.5 NaOH:180 H$_2$O. To this end, TEAH$_3$ and NaOH solution were stirred vigorously at 120 °C. After lowering the temperature to 70 °C, TEOS were slowly added to control silica condensation, and stirred to reach 118 °C. Afterwards, CTABr were added to the solution until completely dissolved, which allowed the incorporation of deionised water, which was vigorously stirred at 70 °C. This step led to the formation of a white suspension, which was aged at 100 °C for 24 h. The obtained solid was washed with deionised water and ethanol until pH 7, and then dried at 70 °C. Finally, the as-synthesised solid was calcined at 550 °C for 5 h to remove the surfactant molecules.

2.3.2 Derivatisation of the bioactive compounds

Eugenol and thymol aldehydes were prepared by preserving the presence of their hydroxyl group given the important role that these hydroxyl moieties play in antifungal activity (Ahmad et al., 2011; Rao, Zhang, Muend, & Rao, 2010). The eugenol aldehyde was obtained by a Reimer-Tiemann reaction. For this purpose, 150 mL of water at 80
°C were used to dissolve 22 mmol of eugenol. Afterwards, the temperature was lowered to 60 °C, and 400 mmol of KOH and 88 mmol of chloroform were added. The last reagent was incorporated at a ratio of 1 mL/h for 7 h due to the exothermic character of this reaction. The reaction mixture was kept at 60 °C for 8 h. Finally, 50 mL of H$_2$SO$_4$ (10% v/v) were added and the mixture was extracted using n-butane. The organic phase was rotavapored at reduced pressure to obtain the eugenol aldehyde.

The thymol aldehyde was synthesised by mixing 40 mmol of thymol, 150 mL of acetonitrile, 150 mmol of trimethylamine and 40 mmol of MgSO$_4$. This mixture was stirred for 15 min at room temperature in an argon atmosphere. Then 270 mmol of paraformaldehyde were added to the mixture and refluxed for 3.5 h at 83 °C. After cooling the solution, it was acidified using 320 mL of HCl (5% v/v) and stirred for 15 min at room temperature in an argon atmosphere. The organic phase was extracted using diethyl ether, and then removing the volatiles at reduced pressure. The reaction yield was calculated by $^1$H NMR in a Bruker AV400 spectrometer (Bruker Daltonik GmbH, Bremen, Germany) which operated at room temperature.

2.3.3 Immobilisation of the bioactive compounds on the surface of MCM-41 microparticles

The immobilisation of the eugenol and thymol aldehydes on the surface of MCM-41 microparticles was carried out through the synthesis of the corresponding alkoxy silane derivatives. The eugenol or thymol aldehyde (2 mL) was mixed with 20 mL of dichloromethane, 2.3 mL (10 mmol) of APTES and MgSO$_4$. The solution was stirred for 1 h at 38 °C in reflux. The mixture was filtered and the organic phase was removed at reduced pressure to obtain the corresponding eugenol or thymol
alkoxysilane derivative. Then 1 g of the MCM-41, 30 mL of acetonitrile and an excess of the corresponding alkoxysilane derivatives were stirred for 5.5 h at room temperature. Solids were filtered, washed with acetonitrile and dried for 24 h at low pressure.

2.3.4 Characterisation of MCM-41 microparticles

The characterisation of the microparticulated MCM-41 (bare and functionalised with eugenol and thymol) was performed by particle size distribution, ζ-potential, field emission scanning electron microscopy (FESEM), thermogravimetric analyses (TGA) and an elemental analysis.

Particle size distribution was determined in deionised water using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK), and applying the Mie theory (refractive index of 1.45, absorption index of 0.1). The ζ-potential analysis was run in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples were diluted with deionised water (1 mg/mL) and sonicated before being measured. The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ-potential values. Particle size distribution and the ζ-potential analysis were performed in triplicate. FESEM images were obtained by a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. Thermogravimetric analyses were carried out on a TGA/SDTA 851e balance (Mettler Toledo, Columbus, USA), from 25 to 1,000 °C with a heating rate of 10 °C/min in an oxidant atmosphere (air, 80 mL/min). An elemental analysis for C, H, and N was performed by a combustion analysis in a CHNOS model Vario EL III (Elemental Analyses System GMHB, Langenselbold, Germany).
2.3.5 Antifungal activity of eugenol and thymol immobilised on the surface of MCM-41 microparticles

The evaluation of the antifungal activity of the immobilised bioactive agents against *A. flavus, A. niger, P. expansum, Z. rouxii* and *Z. bailii* was made by the methodology described in Section 2.2. The concentration of the MCM-41 microparticles functionalised with eugenol or thymol was established based on the thermogravimetric and elemental analyses to add equal amounts of eugenol and thymol (0, 0.1, 0.2, 0.3 and 0.4 mg/mL) to the media than to the free samples. By these means, the comparison of the antifungal activity between the pure and immobilised bioactive agents was made. The elemental analysis data revealed that the content of the eugenol and thymol immobilised on the surface of MCM-41 microparticles was 65 mg/g solid and 76 mg/g solid, respectively. Positive controls were prepared with bare MCM-41 and 1% (w/v) of Tween 80. The results were expressed as log CFU/mL. Each assay was performed in triplicate.

2.4 Study of the influence of MCM-41 microparticles functionalised with eugenol and thymol in strawberry jam

To evaluate the *in vivo* antifungal effect of the bioactive agents immobilised on the surface of MCM-41 microparticles, strawberry jams were prepared and inoculated with two representative microorganisms. *A. niger* was selected as a mould for its frequent isolation in strawberries, while *Z. bailii* was used as a target yeast for its ability to grow in high sugar products (60% w/w) and at a low pH (Stratford, Steels, Nebe-von-Caron, Novodvorska, Hayer, & Archer 2013). The concentrations of the free and immobilised
eugenol and thymol on MCM-41 were selected according to the MFCs determined in the \textit{in vitro} assays.

\subsection*{2.4.1 Jam preparation}

Jam preparation was obtained according to the procedure reported by Ribes et al. (2016). Strawberry jam was obtained by mixing fruit and sugar in a ratio of 65:35 and cooked at 100 °C for 30 min to reach a 60 °Brix in the product as described in the Spanish quality regulation for fruit jam (BOE, 2003). This process was carried out in an electrical food processor (Thermomix TM 31, Vorwerk M.S.L, Spain). The free and immobilised eugenol and thymol, as well as the bare MCM-41 microparticles, were added to 15 g of strawberry jams, once suspended in 1\% (w/v) of Tween 80 in order to ensure the complete distribution of the agents, at ambient temperature and then homogenising adequately by using a sterilised spatula for 1 min.

\subsection*{2.4.2 Antifungal effectiveness in strawberry jam}

The \textit{in vivo} antifungal effectiveness of the free and immobilised bioactive agents against \textit{A. niger} and \textit{Z. bailii} was examined by the methodology described by Ribes et al. (2016). Fifteen grams of strawberry jam (control, control with bare MCM-41 microparticles, free bioactive agents and bioactive agents immobilised on MCM-41) were inoculated with 100 µL of the fungi solution (10⁶ CFU/mL) and incubated at 25 °C for 28 days.

At each analysis day, 10 grams of every sample were placed in sterile plastic bags containing 90 mL of tryptone phosphate water and homogenised for 1 min in a Stomacher blender (Masticator IUL, S.A. Instruments, Germany). Serial dilutions were prepared and 0.1 mL were spread on the surface of the agar plates. Three Petri dishes
were prepared per formulation, microorganism and analysis day, plus the control samples (n=120). *A. niger* and *Z. bailii* counts were done on PDA and YPD agar plates, respectively, after a 72-hour incubation at 25 °C (Pascual & Calderón, 2000). All the assays were performed in triplicate.

2.4.3 Sensory evaluation

A sensory analysis was carried out to evaluate the feasibility of immobilisation to reduce the impact provoked by eugenol and thymol on strawberry jam (ISO 4121: 2003). For this purpose, a panel of 12 trained judges participated in this study. Panellists were trained during preliminary sessions to identify the typical aromas of eugenol and thymol, calibrated using aqueous solutions of these compounds at different concentrations (0, 0.1, 0.2, 0.3 and 0.5 mg of the bioactive compound per g of solution) (ISO 8586: 2012). During the assessment, a 5-point aroma intensity scale was used: from 0, no descriptor, to 5, extremely intense. Each panellist evaluated the intensity aroma of eugenol or thymol on strawberry jam samples, which contained these compounds that were free and immobilised on the MCM-41 surface. The concentrations of the free and immobilised eugenol and thymol were selected according to the MFCs determined in the *in vitro* assays.

In order to quantify the effect of immobilisation to reduce the effect of bioactive compounds on strawberry jam aroma, the rates given to the samples with the immobilised compound were compared to those given to strawberry jam with free eugenol and thymol at the same concentration.
2.5 Statistical analysis

The results obtained in the *in vitro* and *in vivo* tests to evaluate the antifungal activity of the free and immobilised eugenol and thymol were analysed by a multifactor analysis of variance (multifactor ANOVA). The characterisation of the mesoporous silica particles and the sensory analysis were evaluated by a one-way ANOVA. The least significance procedure (LSD) was employed to test for differences between averages at the 5% significance level. Data were statistically processed by Statgraphics Centurion XVI.

3. Results and discussion

3.1 Antifungal activity of free eugenol and thymol

The counts of *A. flavus*, *A. niger* and *P. expansum*, *Z. bailii* and *Z. rouxii* after free eugenol and thymol treatment are shown in Figure 1. Both the bioactive compounds showed significant (*p*<0.05) antifungal activity, which affected fungi growth in a dose-dependent manner. With eugenol, the use of 0.3 mg/mL led to a reduction of between 3 and 5 log-cycles for the *Aspergillus* and *Zygosaccharomyces* genera after 72 h and 48 h, respectively, of its inoculation. The growth inhibition of *A. flavus*, *A. niger*, *Z. bailii* and *Z. rouxii* was achieved by employing 0.4 mg/mL of eugenol (MFC). With *P. expansum*, inhibition was attained by using 0.2 mg/mL of eugenol, and this concentration was the MFC.

When 0.2 mg/mL of thymol were added to the media, a reduction of between 3 and 4 log-cycles took place for the *Aspergillus* and *Zygosaccharomyces* genera after 72 h and 48 h, respectively, of its inoculation. Thymol inhibited the growth of all the target microorganisms tested at 0.4 mg/mL, which corresponded to the MFC value.
It is worth mentioning that the discontinuous horizontal line indicates the initial counts (CFU/mL) (Figure 1). Above this line, no antifungal effect was achieved, while this line indicated the fungistatic activity of free eugenol or thymol. In addition, below the discontinuous line a fungicidal effect is observed. Taking it into account, a significant \((p<0.05)\) fungicidal effect was exhibited when using \(\geq 0.3\) mg/mL of eugenol and thymol for \(A.\ niger\), \(Z.\ bailii\) and \(Z.\ rouxii\), which was stronger for thymol. Indeed, the fungicidal effect of thymol against \(A.\ flavus\) was evidenced when \(0.2\) mg/mL were employed. For \(P.\ expansum\), the treatment with \(0.1\) and \(0.2\) mg/mL of eugenol and thymol, respectively, showed clear fungistatic activity.

The differences in the molecular structure of both the antifungal agents most likely determine their antifungal effectiveness. The hydroxyl group present in thymol is responsible for the strong ability to dissolve and accumulate in the cell membrane, and lead to its destabilisation due to marked proton transfer disruption (Ahmad et al., 2011; Rao et al., 2010). Furthermore, the generally weaker antifungal activity of eugenol at low concentrations could be related to its lower hydrophobicity, and also to the presence of a methoxyl group in orthoposition, which diminished its ability to release a proton from the hydroxyl group (Ben Arfa, Combes, Preziosi-Belloy, Gontard, & Chalier, 2006). Similar results have been obtained by Abbaszadeh, Sharifzadeh, Shokri, Khosravi, and Abbaszadeh (2014) when they applied eugenol as an alternative agent to control fungi development. However, the MFC values of thymol against the \(Aspergillus\), \(Penicillium\) and \(Zygosaccharomyces\) species were lower than the data obtained in this study. Abbaszadeh et al. (2014) showed the influence of thymol with the MFC values of 150 and 250 µg/mL against \(A.\ flavus\) and \(A.\ niger\), respectively. In another study, Monu, Techathuwan, Wallis, Critzer, and Davidson (2016) found that
eugenol and thymol inhibited *Z. bailii* growth at 200 mg/L. The differences between these findings and the results reported herein could be due to the strains selected, the type of assay employed and incubation times used.

3.2 Characterisation of the bare and functionalised MCM-41 microparticles

Antifungal microparticles were prepared by the immobilisation of eugenol and thymol on the surface of the MCM-41 support. In a first step, both bioactive compounds were reacted with APTES to obtain the corresponding trialcoxysilane derivative. The efficiency of the alkoxysilane derivatisation process was evaluated by the $^1$H NMR analysis. For the two bioactive agents, the product yield estimated from the $^1$H NMR spectra was 20-40%. The alkoxysilanes derivatives reacted in a second step with the silanol groups of the MCM-41 microparticles yielded the final functionalised solids.

Bare and functionalised MCM-41 microparticles were characterised by standard techniques. Table 1 summarises the $d_{0.5}$ and the $\zeta$-potential values for the MCM-41 microparticles (bare and immobilised with eugenol and thymol). The bare MCM-41 microparticles showed a $d_{0.5}$ of 3.13±0.14 µm, whereas an increased particle mean diameter was obtained when the particles were functionalised with eugenol and thymol (4.37±0.12 and 4.1±0.2 µm, respectively).

The $\zeta$-potential values of the samples are provided in Table 1. The bare MCM-41 microparticles had negative $\zeta$-potential values (-35.9±1.4). After the immobilisation of eugenol and thymol on the mesoporous material surface, the $\zeta$-potential changed to weak negative or positive values in agreement with the functionalisation of the MCM-41 surface with eugenol and thymol. The change we noted in the $\zeta$-potential values upon functionalisation has also been observed by Pérez-Esteve et al. (2016) in mesoporous
silica supports loaded with folic acid and functionalised with amines, and also by Mathew et al. (2016) in succinamic acid functionalised MCM-41 particles.

Figure 2 shows the FESEM images of the bare and functionalised MCM-41. As seen, no changes on the surface of the mesoporous supports were detected when comparing the bare MCM-41 and the functionalised samples, which confirms that the immobilisation of eugenol and thymol on the surface did not affect the integrity of the mesoporous silica particles. As previously mentioned, the content of the eugenol and thymol immobilised on the surface of the MCM-41 microparticles, obtained from the thermogravimetric and elemental analyses, was 65 mg and 76 mg per gram of solid, respectively. These data were used to calculate the amount of MCM-41 functionalised with eugenol and thymol needed to provide an equivalent dose of bioactive compounds compared to the free molecule (Section 2.3.5).

3.3 Antifungal activity of eugenol and thymol immobilised on the surface of MCM-41 microparticles

The antifungal activity of eugenol and thymol immobilised on the surface of MCM-41 against *A. flavus, A. niger, P. expansum, Z. bailii* and *Z. rouxii* is summarised in Figure 3.

No growth inhibition was observed in any mould and yeast strains in the presence of the bare MCM-41. These results agree with those obtained by Wehling et al. (2013), who also evaluated the antimicrobial activity of bare silica particles. In contrast, mould and yeast growth significantly reduced ($p<0.05$) in the presence of increasing amounts of MCM-41 functionalised with eugenol and thymol. The MCM-41 that contained eugenol as an antifungal agent exhibited greater effectiveness than the thymol
immobilised on the MCM-41 microparticles against all the evaluated fungi species. Growth of *P. expansum*, *Z. bailii* and *Z. rouxii* was inhibited by using 0.2 mg/mL of immobilised eugenol (MFC), whereas the genus *Aspergillus* presented less sensitivity at this concentration. The total inhibition of *A. flavus* and *A. niger* was attained at 0.3 and 0.4 mg/mL of immobilised eugenol, respectively, which were the MFC concentrations.

Conversely, when MCM-41 functionalised with thymol was tested against all the target microorganisms, weak antifungal efficacy was observed at low thymol concentrations. The use of 0.2 mg/mL of immobilised thymol led to a reduction of between 2 and 4 log-cycles, whereas, the immobilised eugenol at the same concentration attained a 5 log reduction for *A. niger* and inhibited fungi development as in the case of *P. expansum*, *Z. bailii* and *Z. rouxii*. As previously mentioned, this tendency was not observed for *A. flavus* (Figure 3). Inhibition of *A. flavus* was observed when using 0.3 mg/mL of thymol immobilised on MCM-41, which corresponds to its MFC value. The MFC values for *A. niger*, *P. expansum*, *Z. bailii* and *Z. rouxii* were observed by using 0.4 mg/g of immobilised thymol.

In addition, a significant (*p<0.05*) fungicidal effect was observed when using ≥ 0.2 mg/mL of eugenol and thymol immobilised on MCM-41 microparticles for *A. niger*, *P. expansum*, *Z. bailii* and *Z. rouxii*, and was stronger for eugenol. Moreover, the fungicidal effect of immobilised eugenol and thymol against *A. flavus* was exhibited when 0.3 mg/mL were utilised (Figure 3).

As far as we know, this is the first study that evaluate the antifungal efficacy of eugenol and thymol derivatives immobilised on the surface of microparticulated MCM-41 against the genera *Aspergillus*, *Penicillium* and *Zygosaccharomyces* have been reported.
When the results of the free and immobilised eugenol and thymol were compared, it was generally established that greater antifungal effectiveness was observed when these compounds were immobilised on the MCM-41 support. This could be due to: i) the intense antifungal activity of the MCM-41 microparticles after eugenol and thymol immobilisation due to the high density of the antifungal compound on the mesoporous material surface; and/or ii) the volatility reduction of bioactive agents when immobilised on the surface of MCM-41 microparticles.

3.4 Study of the influence of MCM-41 microparticles functionalised with eugenol and thymol in strawberry jam

3.4.1 Antifungal effectiveness in strawberry jam

The development of *A. niger* and *Z. bailii* in non-inoculated and inoculated strawberry jams stored at 25 °C for 28 days is shown in Figure 4. As noted, the jams prepared with free and immobilised eugenol exhibited no mould and yeast development throughout the evaluation period. On the contrary, the samples prepared with the free thymol and thymol-MCM-41 did not inhibit the fungal growth of the samples. However, it is noteworthy that the strawberry jams prepared with the free thymol, and inoculated with *A. niger* and *Z. bailii*, exhibited a more fungi development compared to the samples that contained the thymol-MCM-41 microparticles. These results agree with those reported in the *in vitro* antifungal assays (Section 3.3), where the antifungal effectiveness of the MCM-41 that contained the immobilised bioactive agents was enhanced.

Despite the same amount of the free and immobilised thymol being used in the jam samples and culture media against *A. niger* and *Z. bailii*, different antifungal activity
was observed. This could be related to the presence of antagonistic interactions with other ingredients, such as carbohydrates (Pitt & Hocking, 2009). Firouzi, Shekarforough, Nazer, Borumand, and Jooyandeh (2007) reported that despite \textit{in vitro} assays with EOs and their main components suggesting that compounds, such as oregano and nutmeg, displayed substantial antimicrobial activity, the amounts required when used in food systems increased (approx. 1–3% higher).

3.4.2 Sensory evaluation

A sensory evaluation was carried out to test the feasibility of immobilisation to avoid the drawbacks of aromas when incorporating eugenol and thymol into food samples. Average scores of aroma intensity of free and immobilised bioactive agents are shown in Figure 5. Free bioactive agents (eugenol and thymol) incorporated to water samples exhibited the highest aroma intensity score, whereas the aroma of the same compounds in jam samples was perceived in a lower intensity. The immobilisation reduced the aroma intensity of both bioactive compounds, being this reduction higher for eugenol in water samples. In the case of immobilised agents added to jam samples, the scores were the lowest. No significant differences were observed between immobilised eugenol and thymol in jam samples. The comparison of the jam samples with the free and immobilised bioactive compounds established that immobilisation reduced the intensity of eugenol and thymol aromas evaluated by assessors more than 92% and 96%, respectively. The assessment results indicated that, even though immobilisation could not completely eliminate the typical thymol and eugenol aroma in the jam samples, this technique was able to significantly reduce the aroma intensity of these compounds in strawberry jam. Thus, the results confirm the feasibility of
immobilisation as a technique to avoid the impact of eugenol and thymol on the sensory profile of food samples. This promising technique could be used with other substances that are not currently viable given their adverse impact on the sensory perception of applied foods.

4. Conclusion

Free eugenol and thymol exhibit excellent properties as antifungal agents against several mould and yeast strains. When incorporated in their free form, eugenol induces better preservation of strawberry jam in terms of fungal spoilage compared to thymol. However, after immobilisation on MCM-41 microparticles, both bioactive agents have improved the antifungal properties and their impact on jam odour compared to the free compounds are weaker. These results suggest that the use of bioactive agents from plants immobilised on the surface of mesoporous silica materials acts as promising antifungal agents for controlling mould and yeast spoilage, and by diminishing the current industrial limitation due to their strong flavour at the same time.

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**Figure captions**

**Figure 1.** Microbial counts of moulds and yeasts against free eugenol and thymol at 25 °C for 72 h and at 25 °C for 48 h, respectively. Mean value (n=3) ± standard deviation. The discontinuous horizontal line indicates the initial count (CFU/mL).

**Figure 2.** FESEM images of a) bare MCM-41, b) eugenol-MCM-41, c) thymol-MCM-41 microparticles.

**Figure 3.** Microbial counts of moulds and yeasts against immobilised eugenol and thymol at 25 °C for 72 h and at 25 °C for 48 h, respectively. Mean value (n=3) ± standard deviation. The discontinuous horizontal line indicates the initial CFU/mL.

**Figure 4.** Influence of the free and immobilised eugenol and thymol on the growth of (a) *Aspergillus niger* and (b) *Zygosaccharomyces bailii* in inoculated strawberry jams for 28 days at 25 °C. Mean values (n=3) ± standard deviation.

**Figure 5.** Average score of the sensory impact of free and immobilised eugenol and thymol in aqueous media or strawberry jam being 0: not perceived and 5: very high intense. Different letters indicates significant differences between samples (*p*<0.05) (n=12).
Table captions

Table 1. Particle size ($d_{0.5}$) and $\zeta$-potential values of MCM-41 microparticles (bare) and
with eugenol and thymol derivates immobilised on its surface (Eugenol-MCM-41 and
Thymol-MCM-41). Values are expressed as mean (n=3) ± standard deviation.
• Innovative antifungal systems with eugenol and thymol were studied
• Immobilisation of bioactive agents on MCM-41 enhances its antifungal action
• Eugenol and thymol immobilised on MCM-41 improves the drawbacks due to its flavour
• Eugenol and thymol immobilised on MCM-41 are promising antifungal agents
Figure 1
Figure 2
Figure 3
Figure 4

a) Aspergillus niger

b) Zygosaccharomyces bailii
Figure 5
<table>
<thead>
<tr>
<th>Sample</th>
<th>$d_{0.5}$ (µm)</th>
<th>ζ-potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>Bare MCM-41</td>
<td>3.13 ± 0.14 $^a$</td>
<td>-35.9 ± 1.4 $^a$</td>
</tr>
<tr>
<td>Eugenol-MCM-41</td>
<td>4.37 ± 0.12 $^c$</td>
<td>-0.4 ± 0.4 $^b$</td>
</tr>
<tr>
<td>Thymol-MCM-41</td>
<td>4.1 ± 0.2 $^b$</td>
<td>10.8 ± 2.1 $^c$</td>
</tr>
</tbody>
</table>

$^a$, $^b$, $^c$ Different superscripts indicate differences among mesoporous silica materials.