Ribes-Llop, S.; Pérez-Esteve, É.; Ruiz Rico, M.; Fuentes López, A.; Talens Oliag, P.; Martínez-Máñez, R.; Barat Baviera, JM. (2017). Eugenol and thymol immobilised on mesoporous silica-based material as an innovative antifungal system: Application in strawberry jam. Food Control. 81:181-188. doi:10.1016/j.foodcont.2017.06.006

# 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

Yeasts 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 monoterpene 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 $SiO_2$, 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 ($TEAH_3$), tetraethylorthosilicate (TEOS), (3-Aminopropyl)triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butanone, dimethyl sulfoxide (provide all of them by Sigma-Aldrich, Madrid, Spain), acetonitrile, hydrochloric acid (HCl), magnesium sulphate ($MgSO_4$), potassium hydroxide (KOH) and sulfuric acid ($H_2SO_4$) (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 $10^6$ 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 $H_2O$.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 $H_2SO_4$ (10% v/v) were added and the mixture was extracted using n-butanone. 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 alkoxysilane 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

184 alkoxysilane derivative. Then 1 g of the MCM-41, 30 mL of acetonitrile and an excess
185 of the corresponding alkoxysilane derivatives were stirred for 5.5 h at room
186 temperature. Solids were filtered, washed with acetonitrile and dried for 24 h at low
187 pressure.

188 *2.3.4 Characterisation of MCM-41 microparticles*

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

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

208 *2.3.5 Antifungal activity of eugenol and thymol immobilised on the surface of MCM-41*
209 *microparticles*

210 The evaluation of the antifungal activity of the immobilised bioactive agents
211 against *A. flavus, A. niger, P. expansum, Z. rouxii* and *Z. bailii* was made by the
212 methodology described in Section 2.2. The concentration of the MCM-41
213 microparticles functionalised with eugenol or thymol was established based on the
214 thermogravimetric and elemental analyses to add equal amounts of eugenol and thymol
215 (0, 0.1, 0.2, 0.3 and 0.4 mg/mL) to the media than to the free samples. By these means,
216 the comparison of the antifungal activity between the pure and immobilised bioactive
217 agents was made. The elemental analysis data revealed that the content of the eugenol
218 and thymol immobilised on the surface of MCM-41 microparticles was 65 mg/g solid
219 and 76 mg/g solid, respectively.Positive controls were prepared with bare MCM-41 and
220 1% (w/v) of Tween 80. The results were expressed as log CFU/mL. Each assay was
221 performed in triplicate.
222

223 2.4 Study of the influence of MCM-41 microparticles functionalised with eugenol and
224 thymol in strawberry jam

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

231 eugenol and thymol on MCM-41 were selected according to the MFCs determined in
232 the *in vitro* assays.

233 *2.4.1 Jam preparation*

234 Jam preparation was obtained according to the procedure reported by Ribes et al.
235 (2016). Strawberry jam was obtained by mixing fruit and sugar in a ratio of 65:35 and
236 cooked at 100 °C for 30 min to reach a 60 °Brix in the product as described in the
237 Spanish quality regulation for fruit jam (BOE, 2003). This process was carried out in an
238 electrical food processor (Thermomix TM 31, Vorwerk M.S.L, Spain). The free and
239 immobilised eugenol and thymol, as well as the bare MCM-41 microparticles, were
240 added to 15 g of strawberry jams, once suspended in 1% (w/v) of Tween 80 in order to
241 ensure the complete distribution of the agents, at ambient temperature and then
242 homogenising adequately by using a sterilised spatula for 1 min.

243 *2.4.2 Antifungal effectiveness in strawberry jam*

244 The *in vivo* antifungal effectiveness of the free and immobilised bioactive agents
245 against *A. niger* and *Z. bailii* was examined by the methodology described by Ribes et
246 al. (2016). Fifteen grams of strawberry jam (control, control with bare MCM-41
247 microparticles, free bioactive agents and bioactive agents immobilised on MCM-41)
248 were inoculated with 100 µL of the fungi solution ($10^6$ CFU/mL) and incubated at 25
249 °C for 28 days.

250 At each analysis day, 10 grams of every sample were placed in sterile plastic bags
251 containing 90 mL of tryptone phosphate water and homogenised for 1 min in a
252 Stomacher blender (Masticator IUL, S.A. Instruments, Germany). Serial dilutions were
253 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.

277 2.5 Statistical analysis

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

285 **3. Results and discussion**

286 3.1 Antifungal activity of free eugenol and thymol

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

296 When 0.2 mg/mL of thymol were added to the media, a reduction of between 3 and
297 4 log-cycles took place for the *Aspergillus* and *Zygosaccharomyces* genera after 72 h
298 and 48 h, respectively, of its inoculation. Thymol inhibited the growth of all the target
299 microorganisms tested at 0.4 mg/mL, which corresponded to the MFC value.

300 It is worth mentioning that the discontinuous horizontal line indicates the initial
301 counts (CFU/mL) (Figure 1). Above this line, no antifungal effect was achieved, while
302 this line indicated the fungistatic activity of free eugenol or thymol. In addition, below
303 the discontinuous line a fungicidal effect is observed. Taking it into account, a
304 significant ($p<0.05$) fungicidal effect was exhibited when using ≥ 0.3 mg/mL of
305 eugenol and thymol for *A. niger*, *Z. bailii* and *Z. rouxii*, which was stronger for thymol.
306 Indeed, the fungicidal effect of thymol against *A. flavus* was evidenced when 0.2
307 mg/mL were employed. For *P. expansum*, the treatment with 0.1 and 0.2 mg/mL of
308 eugenol and thymol, respectively, showed clear fungistatic activity.

309 The differences in the molecular structure of both the antifungal agents most likely
310 determine their antifungal effectiveness. The hydroxyl group present in thymol is
311 responsible for the strong ability to dissolve and accumulate in the cell membrane, and
312 lead to its destabilisation due to marked proton transfer disruption (Ahmad et al., 2011;
313 Rao et al., 2010). Furthermore, the generally weaker antifungal activity of eugenol at
314 low concentrations could be related to its lower hydrophobicity, and also to the presence
315 of a methoxyl group in orthoposition, which diminished its ability to release a proton
316 from the hydroxyl group (Ben Arfa, Combes, Preziosi-Belloy, Gontard, & Chalier,
317 2006). Similar results have been obtained by Abbaszadeh, Sharifzadeh, Shokri,
318 Khosravi, and Abbaszadeh (2014) when they applied eugenol as an alternative agent to
319 control fungi development. However, the MFC values of thymol against the
320 *Aspergillus, Penicillium* and *Zygosaccharomyces* species were lower than the data
321 obtained in this study. Abbaszadeh et al. (2014) showed the influence of thymol with
322 the MFC values of 150 and 250 µg/mL against *A. flavus* and *A. niger*, respectively. In
323 another study, Monu, Techathuvanan, 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 ζ-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 ζ-potential values of the samples are provided in Table 1. The bare MCM-41 microparticles had negative ζ-potential values (-35.9±1.4). After the immobilisation of eugenol and thymol on the mesoporous material surface, the ζ-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 ζ-potential values upon functionalisation has also been observed by Pérez-Esteve et al. (2016) in mesoporous

348 silica supports loaded with folic acid and functionalised with amines, and also by
349 Mathew et al. (2016) in succinamic acid functionalised MCM-41 particles.

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

360 3.3 Antifungal activity of eugenol and thymol immobilised on the surface of MCM-41
361 microparticles

362 The antifungal activity of eugenol and thymol immobilised on the surface of MCM-
363 41 against *A. flavus*, *A. niger*, *P. expansum*, *Z. bailii* and *Z. rouxii* is summarised in
364 Figure 3.

365 No growth inhibition was observed in any mould and yeast strains in the presence
366 of the bare MCM-41. These results agree with those obtained by Wehling et al. (2013),
367 who also evaluated the antimicrobial activity of bare silica particles. In contrast, mould
368 and yeast growth significantly reduced ($p<0.05$) in the presence of increasing amounts
369 of MCM-41 functionalised with eugenol and thymol. The MCM-41 that contained
370 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

418 was observed. This could be related to the presence of antagonistic interactions with
419 other ingredients, such as carbohydrates (Pitt & Hocking, 2009). Firouzi,
420 Shekarforoush, Nazer, Borumand, and Jooyandeh (2007) reported that despite *in vitro*
421 assays with EOs and their main components suggesting that compounds, such as
422 oregano and nutmeg, displayed substantial antimicrobial activity, the amounts required
423 when used in food systems increased (approx. 1–3% higher).
424
425 *3.4.2 Sensory evaluation*

426 A sensory evaluation was carried out to test the feasibility of immobilisation to
427 avoid the drawbacks of aromas when incorporating eugenol and thymol into food
428 samples. Average scores of aroma intensity of free and immobilised bioactive agents are
429 shown in Figure 5. Free bioactive agents (eugenol and thymol) incorporated to water
430 samples exhibited the highest aroma intensity score, whereas the aroma of the same
431 compounds in jam samples was perceived in a lower intensity. The immobilisation
432 reduced the aroma intensity of both bioactive compounds, being this reduction higher
433 for eugenol in water samples. In the case of immobilised agents added to jam samples,
434 the scores were the lowest. No significant differences were observed between
435 immobilised eugenol and thymol in jam samples. The comparison of the jam samples
436 with the free and immobilised bioactive compounds established that immobilisation
437 reduced the intensity of eugenol and thymol aromas evaluated by assessors more than
438 92% and 96%, respectively. The assessment results indicated that, even though
439 immobilisation could not completely eliminate the typical thymol and eugenol aroma in
440 the jam samples, this technique was able to significantly reduce the aroma intensity of
441 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.

## Acknowledgements

Authors gratefully acknowledge the financial support from MINECO-Spanish Government and FEDER-EU (Projects AGL2012-39597-C02-01, AGL2015-70235-C2-1-R and MAT2015-64139-C4-1) and the Generalitat Valencia (Project PROMETEOII/2014/047). Furthermore, author Susana Ribes is grateful to the Universitat Politècnica de València (UPV) for her FPI grant.

## References

Abbaszadeh, S., Sharifzadeh, A., Shokri, H., Khosravi, A. R., & Abbaszadeh, A. (2014). Antifungal efficacy of thymol, carvacrol, eugenol and menthol as alternative

agents to control the growth of food-relevant fungi. *Journal de Mycologie Médicale, 24*, 51-56.

Ahmad, A., Khan, A., Akhtar, F., Yousuf, S., Xess, I., Khan, L. A., et al. (2011). Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against *Candida*. *European Journal of Clinical Microbiology & Infectious Diseases, 30*, 41-50.

Barahona, F., Ojea-Jimenez, I., Geiss, O., Gilliland, D., & Barrero-Moreno, J. (2016). Multimethod approach for the detection and characterization of food-grade synthetic amorphous silica nanoparticles. *Journal of Chromatography A, 1432*, 92-100.

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., & Chalier, P. (2006). Antimicrobial activity of carvacrol related to its chemical structure. *Letters in Applied Microbiology, 43(2),* 149–154

Bernardos, A., & Kourimska, L. (2013). Applications of mesoporous silica materials in food - a review. *Czech Journal of Food Sciences, 31(2)*, 99-107.

BOE 2003. Real Decreto 863/2003, de 4 de Julio, por el que se aprueba la Norma de calidad para la elaboración, comercialización y venta de confituras, jaleas, "marmalades" de frutas y crema de castañas (2003).

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods – A review. *International Journal of Food Microbiology, 94*, 223–253.

CFR (2015). Code of federal regulations. Title 21, Part 182.20. Essential oils, oleoresins (solvent-free), and natural extractives (including distillates) that are generally recognized as safe for their intended use.

Chang, Y., McLandsborough, L., & McClements, D. J. (2015). Fabrication, stability and efficacy of dual-component antimicrobial nanoemulsions: Essential oil (thyme oil) and cationic surfactant (lauric alginate). *Food Chemistry, 172*, 298-304.

Dündar-Tekkaya, E., & Yürüm, Y. (2016). Mesoporous MCM-41 material for hydrogen storage: A short review. *International Journal of Hydrogen Energy, 41*, 9789-9795.

Firouzi, R., Shekarforoush, S. S., Nazer, A. H. K., Borumand, Z., & Jooyandeh, A. R. (2007). Effects of essential oils of oregano and nutmeg on growth and survival of *Yersinia enterocolitica* and *Listeria monocytogenes* in barbecued chicken. *Journal of Food Protection, 70*, 2626-2630.

Gammariello, D., Conte, A., Lucera, A., Mastromatteo, M., & Del Nobile, M. A. (2014). Anti-yeast activity of natural compounds: *In vitro* and *in vivo* tests. *Food packaging and shelf life, 1*, 30-37.

Kirsch, K., Tseremmadmid, R., & Vágvölgyi, C. (2011). Essential oils against yeasts and moulds causing food spoilage. In: A., Méndez-Vilas (Eds.), Science against microbial pathogens: communicating current research and technological advances (pp. 1135-1142). Formatex, Badajoz, Spain.

Klarić, M. Š., Kosalec, I., Mastelić, J., Piecková, E., & Pepeljnak, S. (2006). Antifungal activity of thyme (*Thymus vulgaris L.*) essential oil and thymol against moulds from damp dwellings. *Letters in Applied Microbiology, 44*, 36-42.

Manso, S., Cacho-Nerin, F., Becerril, R., & Nerín, C. (2013). Combined analytical and microbiological tools to study the effect on *Aspergillus flavus* of cinnamon essential oil contained in food packaging. *Food Control, 30*, 370-378.

509 Mathew, A., Parambadath, S., Barnabas, M. J., Song, H. J., Kim, J-S., Park, S. S., et al.
510 (2016). Rhodamine 6G assisted adsorption of metanil yellow over succinamic acid
511 functionalized MCM-41. *Dyes and Pigments, 131,* 177-185.

512 Mihai, A. L., & Popa, M. E. (2015). *In vitro* Activity of Natural Antimicrobial
513 Compounds against *Aspergillus* Strains. “ST26733”, International Conference
514 "Agriculture for Life, Life for Agriculture". *Agriculture and Agricultural Science*
515 *Procedia, 6,* 585 – 592.

516 Monu, E. A., Techathuvanan, C., Wallis, A., Critzer, F. J., & Davidson, P. D. (2016).
517 Plant essential oils and components on growth of spoilage yeasts in microbiological
518 media and a model salad dressing. *Food Control, 65,* 73-77.

519 Pascual, M., & Calderón, V. (2000). Hongos. In: S. A. Díaz de Santos (Eds.),
520 *Microbiología Alimentaria. Metodología Analítica para Alimentos y Bebidas* (pp.
521 141-146). Madrid, España.

522 Pérez-Esteve, E., Fuentes, A., Coll, C., Acosta, C., Bernardos, A., Amorós, P., et al.
523 (2015ª). Modulation of folic acid bioaccesibility by encapsulation in pH-responsive
524 gated mesoporous silica particle. *Microporous and Mesoporous Materials, 15,* 124-
525 132.

526 Pérez-Esteve, É., Ruiz-Rico, M., Martínez-Máñez, R., & Barat, J. M. (2015b).
527 Mesoporous Silica-Based Supports for the Controlled and Targeted Release of
528 Bioactive Molecules in the Gastrointestinal Tract. *Journal of Food Science, 80(11),*
529 2504-2516.

530 Pérez-Esteve, E., Ruiz-Rico, M., Fuentes, A., Marcos, M. D., Sancenón, F., Martínez-
531 Máñez, R., et al. (2016). Enrichment of stirred yogurts with folic acid encapsulated

in pH-responsive mesoporous silica particles: Bioaccessibility modulation and physico-chemical characterization. *LWT - Food Science and Technology*, *72*, 351-360.

Pitt, J. I., & Hocking, A. D. (2009). *Fungi and food spoilage*. (3rd ed). Springer: New York, (Chapter 1).

Rao, A., Zhang, Y., Muend, S., Rao, R. (2010). Mechanism of Antifungal Activity of Terpenoid Phenols Resembles Calcium Stress and Inhibition of the TOR Pathway. *Antimicrobial Agents and Chemotherapy, 54*, 5062-5069.

Ribes, S., Fuentes, A., Talens, P., & Barat, J. M. (2016). Use of oil-in-water emulsions to control fungal deterioration of strawberry jams. *Food Chemistry, 211*, 92-99.

Stratford, M., Steels, H., Nebe-von-Caron, G., Novodvorska, M., Hayer, K., & Archer, D. B. (2013). Extreme resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces bailii. International Journal of Food Microbiology, 166*, 126-134.

UNE-EN ISO 4121 (2003). *Sensory analysis. Guidelines for the use of quantitative response scales.* AENOR.

UNE-EN ISO 8586 (2012). *Sensory analysis. General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors*. AENOR.

Wehling, J., Volkmann, E., Grieb, T., Rosenauer, A., Maas, M., Treccani, L., et al. (2013). A critical study: Assessment of the effect of silica particles from 15 to 500 nm on bacterial viability. *Environmental Pollution, 176*, 292-299.

Zabka, M., & Pavela, R. (2013). Antifungal efficacy of some natural phenolic compounds against significant pathogenic and toxinogenic filamentous fungi. *Chemosphere, 93,* 1051-1056.

**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 ζ-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

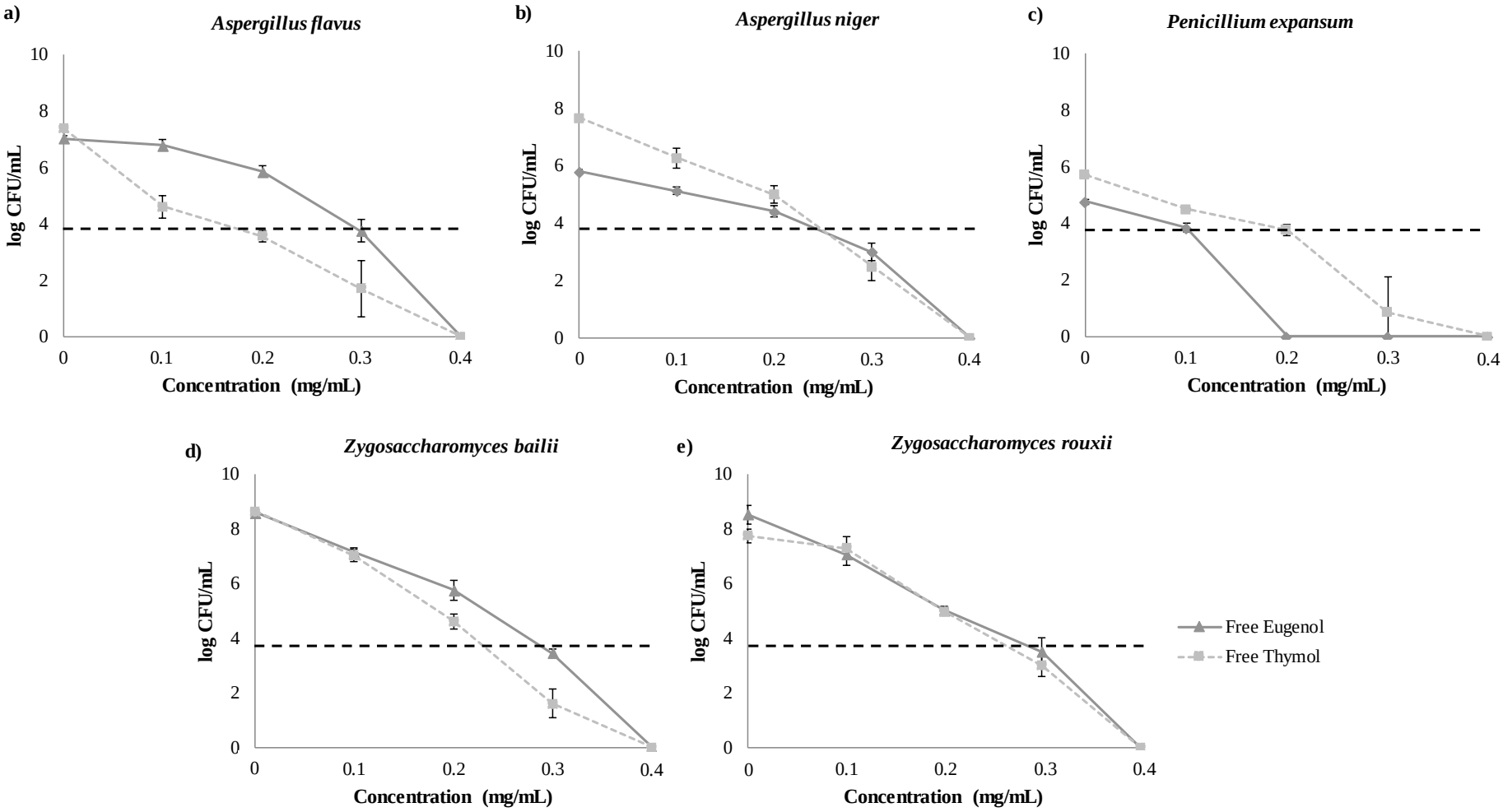

a)
Aspergillus flavus
b)
Aspergillus niger
c)
Penicillium expansum
d)
Zygosaccharomyces bailii
e)
Zygosaccharomyces rouxii
log CFU/mL
Concentration (mg/mL)
0
2
4
6
8
10
0
0.1
0.2
0.3
0.4
Free Eugenol
Free Thymol

**Figure 1**

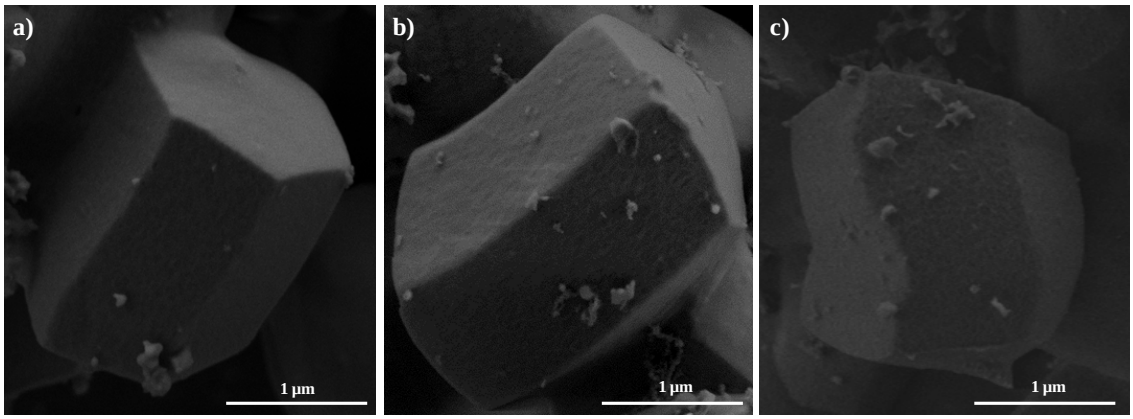



**Figure 2**

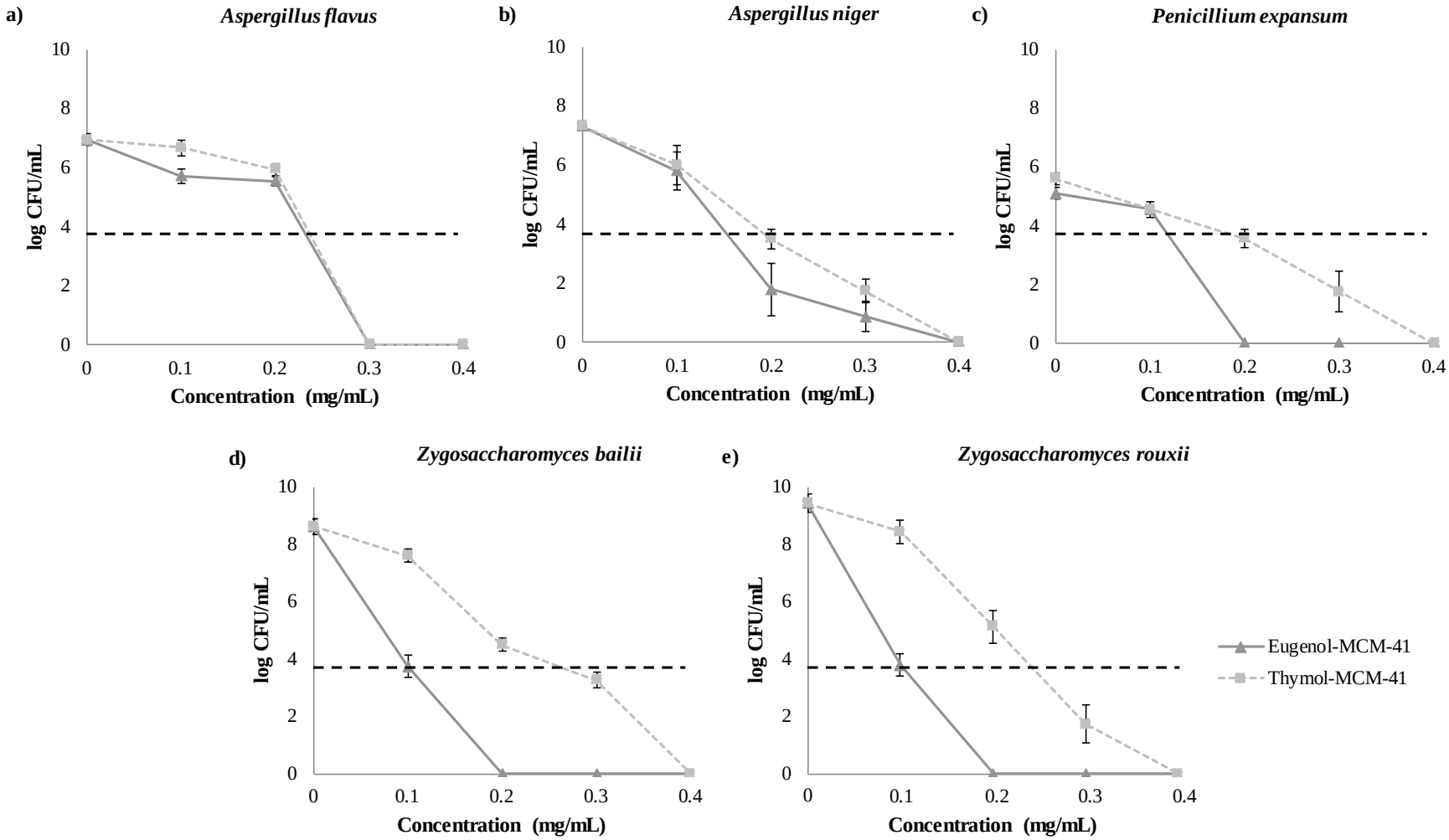
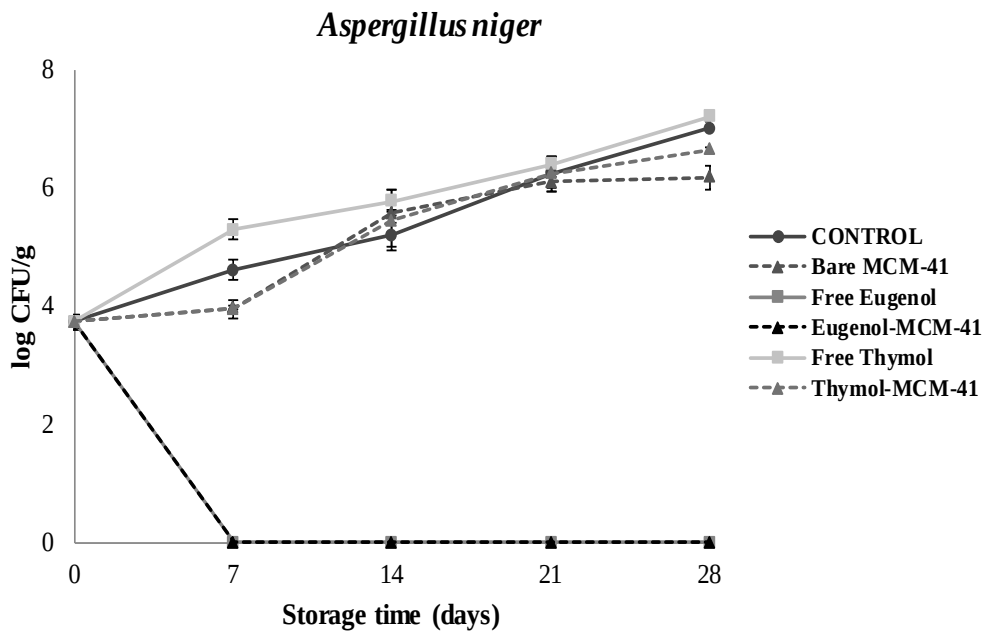

a)
Aspergillus flavus
b)
Aspergillus niger
c)
Penicillium expansum
d)
Zygosaccharomyces bailii
e)
Zygosaccharomyces rouxii
log CFU/mL
Concentration (mg/mL)
0
2
4
6
8
10
0
0.1
0.2
0.3
0.4
Eugenol-MCM-41
Thymol-MCM-41

**Figure 3**

a)


Aspergillus niger
log CFU/g
Storage time (days)
CONTROL
Bare MCM-41
Free Eugenol
Eugenol-MCM-41
Free Thymol
Thymol-MCM-41

b)

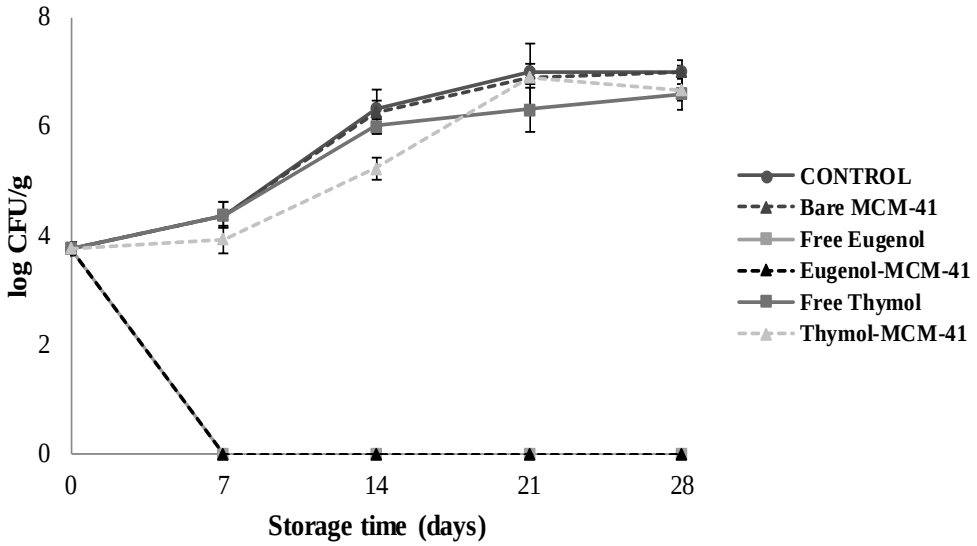

Zygosaccharomyces bailii
log CFU/g
Storage time (days)
CONTROL
Bare MCM-41
Free Eugenol
Eugenol-MCM-41
Free Thymol
Thymol-MCM-41

**Figure 4**

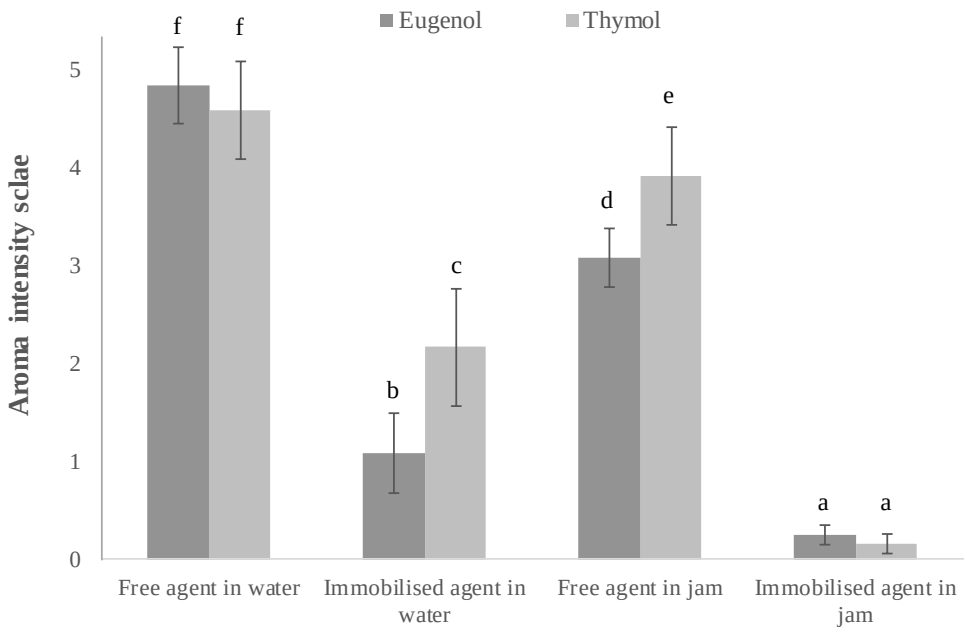

Eugenol
Thymol
Aroma intensity sclae
0
1
2
3
4
5
f
f
b
c
d
e
a
a
Free agent in water
Immobilised agent in water
Free agent in jam
Immobilised agent in jam

**Figure 5**

**Table 1**

| Sample | $d_{0.5}$ (μm) | ζ-potential (mV) |
|---|---|---|
| Bare MCM-41 | 3.13 ± 0.14 [a] | -35.9 ± 1.4 [a] |
| Eugenol-MCM-41 | 4.37 ± 0.12 [c] | -0.4 ± 0.4 [b] |
| Thymol-MCM-41 | 4.1 ± 0.2 [b] | 10.8 ± 2.1 [c] |

[a, b, c] *Different superscripts indicate differences among mesoporous silica materials.*