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Enhancing the antimicrobial activity of eugenol, carvacrol and vanillin immobilised on silica supports against *Escherichia coli* or *Zygosaccharomyces rouxii* in fruit juices by their binary combinations

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Abstract

In this work, the antimicrobial activity and synergistic effect of eugenol, carvacrol and vanillin immobilised on MCM-41 microparticles against *Escherichia coli* and *Zygosaccharomyces rouxii*, on culture media and fruit juices, were investigated. Immobilised bioactive compounds exhibited great *in vitro* antimicrobial activity against the target microorganisms. Moreover, immobilised eugenol inhibited *E. coli* growth after its incorporation into grape juice, regardless of the tested concentration and storage time. Conversely, immobilised carvacrol and eugenol exhibited the greatest effectiveness in reducing fungi development in fruit juices. The immobilised eugenol and vanillin combination exhibited a non-interactive effect (1 < fractional inhibitory concentration index (FICi) ≤4) against *E. coli*. For *Z. rouxii*, a synergistic effect (FICi ≤0.5) between immobilised eugenol and carvacrol was observed in apple juice, whereas an additive effect (0.5 <FICi ≤1) was achieved by mixing these antimicrobial supports in grape juice. This work demonstrates the feasibility of combining functionalised antimicrobial devices to enhance the microbial stabilisation of fruit juices.

Keywords: eugenol; carvacrol; vanillin; mesoporous silica particles; juice.

1. Introduction

Escherichia coli is an acid-tolerant pathogen that can grow up under refrigeration conditions (Mazzotta, 2001; Yuste & Fung, 2004), and has been implicated in outbreaks that involve fresh products, such as fruit juices (Keller & Miller, 2005). Moreover, yeasts from the genus *Zygosaccharomyces* are the most frequently implicated in spoilage of sugar rich-products such as fruit juices (Rojo et al., 2015). Conventional techniques to avoid these problems involves pasteurisation and sterilisation. Despite these thermal treatments are effective to preserve food products, they modify sensory properties and nutritional values (Choi & Nielsen, 2005). Thus, new techniques to preserve commercial juices, such as the addition of chemical preservatives and/or filtrations are being studied. However, no successful results have been reported yet since both microorganisms can survive and proliferate in food products with low pH and water activity values under refrigeration conditions (Rojo et al., 2017).

As consumers demand very fresh and slightly processed products free of chemical additives, the use of natural bioactive compounds with antimicrobial activity has increased in recent years (Ribes, Fuentes, Talens, & Barat, 2018). The antimicrobial activity of several plant-derived compounds, such as eugenol, carvacrol and vanillin, has been demonstrated (Monu, Techathuvanan, Wallis, Critzer, & Davidson, 2016). However given the lipophilic character of these bioactive compounds, their contact with microorganisms in high moisture-content foods is limited. Several authors have reported that higher concentrations of essential oils and/or bioactive compounds are needed to attain the same effectiveness *in vitro* as *in vivo* tests (Burt, 2004) by increasing the non-representative off-flavours of foods due to their strong aroma.

Covalent immobilisation of bioactive compounds on the surface of inert supports has been recently proposed to prevent the above-mentioned limitations (Ruiz-Rico et

al., 2017). MCM-41 particles are porous siliceous materials with a large surface that can react with organic molecules and produce hybrid organic-inorganic systems where organic molecules create a functional layer on their surface (García-Ríos, Ruiz-Rico, Guillamón, Pérez-Esteve, & Barat, 2018). Recently, bioactive compounds immobilised on the surface of MCM-41 microparticles have been used as a potential alternative to improve the antimicrobial activity of free bioactive compounds and to cushion their impact in food commodities (Ribes et al., 2017; Ruiz-Rico et al., 2017). However, the synergistic effect of combining immobilised bioactive compounds to prevent microbial growth and its influence on the food matrix has not been investigated yet. Therefore, the aim of the present work was to evaluate the antimicrobial effectiveness and synergistic effect of eugenol, carvacrol and vanillin immobilised on mesoporous silica microparticles (MCM-41) against *Escherichia coli* and *Zygosaccharomyces rouxii*, and to confirm their antimicrobial activity on fruit juices.

2. Material and Methods

2.1 Strains, media and reagents

Escherichia coli K12 (CECT 433) and *Zygosaccharomyces rouxii* (CECT 1229) were purchased from the Spanish Type Culture Collection (CECT, Burjassot, Spain). This *E. coli* strain was selected because it is a non-pathogenic surrogate of *E. coli* O157:H7, which is recognised as an important cause of foodborne diseases associated with the consumption of acidic foods such as fruit juices (Mazzotta et al., 2001; Mok, Pyatkovskyy, Yousef, & Sastry, 2019). Moreover, the yeast was identified as target microorganism due to its implication in the spoilage of different sugar rich-products like fruit juices (Rojo et al., 2015).

Tryptic Soy Broth (TSB), Tryptone Bile Glucuronic Agar (TBX), Plate Count Agar (PCA), Yeast Peptone Dextrose broth (YPDB) and Nutrient Agar were supplied by Scharlab S.A. (Barcelona, Spain). Eugenol (99% w/w) and carvacrol (≥ 99% w/w) were purchased from Sigma-Aldrich, Madrid, Spain) and vanillin (> 99% w/w) was provided by Ernesto Ventós S.A. (Barcelona, Spain). For the synthesis of the antimicrobial systems, *N*-cetyltrimethylammonium bromide (CTABr), NaOH, triethanolamine ($TEAH_3$), tetraethylorthosilicate (TEOS), (3-Aminopropyl)triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butanone, dimethyl sulfoxide, sodium borohydride and Tween 80 were supplied by Sigma-Aldrich (Madrid, Spain), and acetonitrile, HCl, $MgSO_4$, KOH and H_2SO_4 were purchased from Scharlab S.A. (Barcelona, Spain).

UHT processed apple and grape juices for the *in vivo* antimicrobial assays were bought in a local market.

2.2 Synthesis of MCM-41 microparticles functionalised with bioactive compounds

The MCM-41 microparticles were prepared by the so-called "atrane route" (Ribes et al., 2017). The molar ratio of reagents was set 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. Next, CTABr was incorporated to the solution until completely dissolved allowing the addition of deionised water, which was vigorously stirred at 70 °C. This led to the formation of a white suspension that 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.

Modification of the structure of eugenol and carvacrol was necessary prior to them being anchored to the silica supports. Aldehyde derivatives of these bioactive compounds were synthesised to add a second reactive moiety and maintain the presence of the hydroxyl group responsible of the antimicrobial activity of the bioactive compounds. Eugenol aldehyde was synthesised following a Reimer–Tiemann reaction and carvacrol aldehyde was prepared by direct formylation using paraformaldehyde according to Chen et al. (2009). The aldehydes of eugenol, carvacrol and pure vanillin were reacted with APTES to obtain the corresponding alkoxysilane derivatives. Then these derivatives were immobilised on the surface of the MCM-41 microparticles. After the bioactive compounds were anchored to the particles an stabilisation of the chemical bond of the immobilised compounds was performed according to the methodology described by García-Ríos et al. (2018).

2.3 Materials characterisation

Bare and functionalised supports were characterised by standard techniques to establish their morphology and size, textural properties, surface charge and degree of functionalisation. Particle size was determined in deionised water with a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) by the Mie theory (refractive index of 1.45, absorption index of 0.1). The N_2 adsorption-desorption isotherms were recorded with a Micrometrics ASAP2010 automated sorption analyser (Micromeritics Instrument Corporation, Norcross, USA). Sample was degassed at 90 ºC in vacuum overnight. Specific surface area was calculated from the adsorption data within the low pressure range by the BET model. Pore size was determined following the BJH method. The ζ-potential or zeta potential was measured in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) to establish the surface charge of

the supports. Samples were diluted with deionised water (1 mg/mL) and sonicated before being measured. The Smoluchowsky mathematical model was employed to convert the electrophoretic mobility measurements into ζ-potential values. Particle size distribution and the ζ-potential analysis were conducted in triplicate. The results of the particle size analysis were expressed as $d_{0.5}$. Field emission scanning electron microscopy (FESEM) images were obtained by SUPRA® 25 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. The degree of functionalisation was established by thermogravimetric and elemental analyses. Thermogravimetric analyses were carried out on a TGA/SDTA 851e balance (Mettler Toledo, Columbus, USA), from 25 to 1000 °C with a heating rate of 10 °C/min in an oxidant atmosphere (air, 80 mL/min). An elemental analysis for C, H, O and N was performed by a combustion analysis in a CHNOS model Vario EL III (Elemental Analyses System GMHB, Langenselbold, Germany). The amount of bioactive compounds was calculated by an equation system from the percentage of C and N of the solids and the molecular structure of the bioactive compounds and APTES.

2.4 Antimicrobial activity of the functionalised MCM-41 microparticles

With the purpose of enabling the comprehension of this work, Figure 1 presents the summary of the assays carried out to investigate the antimicrobial activity of the naturally-occurring antimicrobial compounds immobilised on MCM-41 microparticles in a broth and fruit juices and to study the influence of their incorporation on the food matrix.

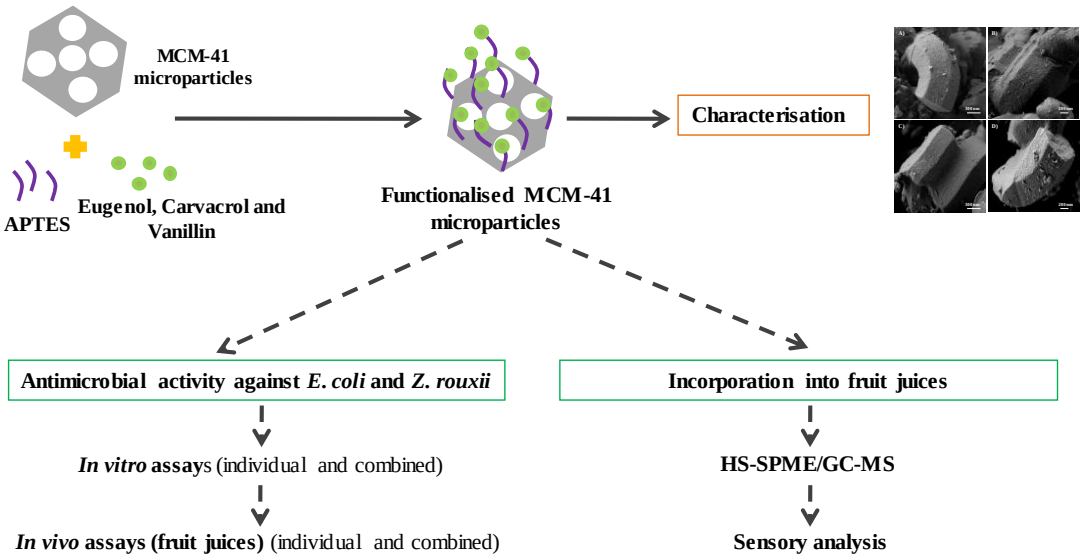

Figure 1. Summary of the assays performed to evaluate the antimicrobial properties and the influence on food matrix of the naturally-occurring antimicrobial compounds immobilised on MCM-41 microparticles.

2.4.1 In vitro antimicrobial activity of the immobilised eugenol, carvacrol and vanillin

The individual antimicrobial activity of the immobilised bioactive compounds was assessed against *E. coli* and *Z. rouxii*. Different concentrations of the immobilised bioactive compounds were tested: 0.025, 0.050, 0.125 and 0.250 mg bioactive compound/mL. The amount of functionalised particles needed to provide these concentrations was calculated by considering the thermogravimetric and elemental analyses results (see section 3.1). The bacterial strain was reconstituted accordingly to the CECT instructions. After that, the bacterial stock was maintained at 4 °C in PCA before its use. The cells of an *E. coli* colony developed on PCA were transferred to 10 mL of TSB and incubated at 37 °C for 24 h to achieve an inoculum with a density of approximately 1×10^8 cells/mL of the TSB. Then, two decimal dilutions in TSB were prepared to achieve a microbial density of 1×10^6 cells/mL. Next, the minimum inhibitory concentration (MIC) of the immobilised bioactive compounds were obtained

by macrodilution (CLSI, 2007) in Erlenmeyer flasks containing 15 mL of TSB and different amount of the supports. The flasks were inoculated with 10 μL of the bacteria suspension (1×10^6 cells/mL of the TSB) to obtain a final concentration of 1×10^4 cells/mL. Flasks were incubated at 37 °C and 180 rpm for 24 h. Then, serial dilutions were made and 100 μL-aliquots of the dilutions were spread on TBX. The MIC was established as the concentration of each immobilised bioactive compound that caused the 90% *in vitro* inhibition of *E. coli* in 2-hour, 6-hour and 24-hour time intervals. The MBC was defined as the lowest concentration of the antimicrobial compound that could kill 99.9% of *E. coli* cells. Positive control (TBX with Tween 80, bare MCM-41 microparticles and the inoculum) and negative control (TBX with Tween 80 and bare microparticles) were used.

The *Z. rouxii* strain was grown in YPDB at 25 °C for 48 h in an orbital shaking incubator at 180 rpm. The yeast cells developed were transferred to test tubes containing 10 mL of sterile water and Tween 80 (0.1% w/v). Afterward, cell suspensions of 10^6 CFU/mL were prepared and confirmed by using a hematocytometer. The MIC and minimum fungicidal concentration (MFC) of the immobilised active compounds were evaluated as described by Ribes et al. (2017) with minor modifications, by inoculating 10 μL of the yeast suspension in 15 mL of YPDB (initial yeast load of 1×10^4 cells/mL). Positive control (YPDB with Tween 80, bare MCM-41 microparticles and the inoculum) and negative control (YPDB with Tween 80 and bare microparticles) were also prepared. All the assays were run in triplicate and the results were expressed as log CFU/mL.

2.4.2 Antimicrobial activity of the immobilised bioactive compounds incorporated into fruit juices

The *in vivo* antimicrobial activity of the immobilised bioactive compounds was determined by using commercial UHT processed apple and grape juices acquired in a local supermarket. The immobilised bioactive compounds used for conducting this test were selected according to the results achieved in Section 2.4.1. The ensued method was similar to that defined in Section 2.4.1. Erlenmeyer flasks were incubated at 8 °C (by simulating refrigeration temperature) for 24 h (*E. coli*) or 48 h (*Z. rouxii*) in a shaking incubator. The MIC and MBC or MFC (bacterium and yeast, respectively) values were determined. Besides, two physicochemical parameters of the juices were determined to establish their influence on the survival of the studied microorganisms. A pH-meter Crison micropH 2001 (Crison Instruments S.A., Alella, Barcelona, Spain) was used to measure the pH value of the samples. Soluble solids (°Brix) determination was performed with a refractometer RFM300 (Bellingham & Stanley Ltd, Kent, United Kingdom).

2.4.3 In vitro and in vivo antimicrobial activity of the immobilised bioactive compounds combinations

In vitro and *in vivo* combination assays were carried out to investigate the synergistic interactions between immobilised bioactive compounds. The concentration range of the immobilised bioactive compounds tested against these strains was 0.025-0.25 mg/mL for eugenol and vanillin (*E. coli*) or for eugenol and carvacrol (*Z. rouxii*) in ratio of 1:1, 1:2, 1:5, 2:1 and 5:1. These combinations were established by taking into account the higher antimicrobial activity of the immobilised eugenol (individually tested) compared to the effectiveness exhibited by carvacrol or vanillin. The antimicrobial procedure was identical to that previously explained to determine individual antimicrobial assays (Sections 2.4.1 and 2.4.2).

The antimicrobial combinations were expressed as a fractional inhibitory concentration index (FICi), which represents the sum of the fractional inhibitory concentrations (FICs) of each studied functionalised bioactive agent (Equations 1 and 2):

$$FIC\ A = (MIC\ of\ A\ in\ the\ presence\ of\ B)/(MIC\ of\ A) \quad (1)$$

$$FIC\ B = (MIC\ of\ B\ in\ the\ presence\ of\ A)/(MIC\ of\ B) \quad (2)$$

where A and B were the respective antimicrobial compounds examined.

The results were interpreted as follows: synergistic effect (FICi ≤ 0.5); additive effect ($0.5 < FICi \leq 1$); non-interactive effect ($1 < FICi \leq 4$); antagonistic effect ($FICi > 4$) (Krisch, Tserennadmid, & Vágvölgyi, 2011; Shi et al., 2017).

2.5 Headspace solid-phase micro extraction and gas-chromatography mass spectrometry (HS-SPME/GC-MS)

In order to verify that the proposed covalent anchoring strategy is able to avoid the volatility of the essential oil components and thus, to hide their strong aroma, the headspace solid-phase micro extraction technique (HS-SPME) coupled to gas-chromatography mass spectrometry (GC-MS) was used. To this end, the fruit juices that contained the equivalent amounts of the free and immobilised bioactive compounds were added to a headspace 10 mL vial, sealed with a PTFE-faced silicone septum (Supelco, Bellefonte, PA). Vials were maintained at 37 °C and vapour was withdrawn from the vial by fibre assembly 65 μm PDMS/DVB Stable Flex Fiber (Supelco, Bellefonte, USA) and injected into the chromatographic column via a transfer line. Bioactive compounds were analysed by an Agilent Technologies 6890N Network Gas Chromatography System equipped with an Agilent Technologies 5973 inert mass

selective detector (Agilent Technologies, Santa Clara, CA, USA), fitted with an HS-5MS column (30 m × 0.25 mm × 0.25 μm film thickness, 5% phenil-methyl silox; Agilent Technologies) using helium as the carrier gas. The chromatography run was started by warming the column at 40 °C for 1 min, and then heated it to 200 °C at a rate of 10 °C/min, with 1-minute hold time. Then it was increased to 250 °C at a rate 15 °C/min, and was held at 250 °C for 3 min. The MS transfer line temperatures were set at 250 °C and 230 °C, respectively. The parameters for the MS analysis were the EI Ion source, electron energy 70 eV, solvent delay 3 min and m/z 40–550 *amu*. Bioactive compounds were identified by matching the mass spectra with the standard mass spectra from the NIST MS Search 2.0 library. The analysis was repeated 3 times for each sample.

The results (peak area) of the fruit juices with the free bioactive compounds (A_1) were compared to the peak area of those with the immobilised bioactive compound (A_2). The aroma reduction (AR) of each compound in each sample was calculated according to Equation 3:

$$AR\ (\%) = ((A_1-A_2)/A_1) \times 100 \qquad (3)$$

2.6 Sensory evaluation

A sensory analysis was performed to evaluate the aroma acceptance of the juices that contained the combinations of the immobilised bioactive compounds. The concentrations of the immobilised antimicrobials were chosen according to the *in vivo* assays results, including equivalent concentrations of the free bioactive compounds. The sensory evaluation was performed by a semi-trained panel of 30 participants, selected from the personnel at the Food Technology Department of the Universitat Politècnica de València. The participants were between 25 and 50 years old and consisted of 18

females and 12 males. They were recruited due to their interest and availability, following the general guidelines UNE-ISO 8586:2012. Training sessions were carried out in order to introduce the panellists to the sensory analysis and to identify and score the quality attributes that describes the samples. Tests were conducted on a 9-point hedonic scale (1 = dislike very much, 9 = like very much) (UNE-ISO 4121:2003). Four sensory parameters were evaluated (appearance, typical colour, typical aroma and general acceptance) and coded samples were given to the panellists at room temperature in a transparent plastic glass.

2.7 Statistical analysis

The results obtained in the *in vitro* and *in vivo* antimicrobial assays of the immobilised bioactive compounds were determined by a multifactor analysis of variance (multifactor ANOVA) to evaluate the effect of concentration, type of bioactive agent and incubation (*in vitro*) or storage (*in vivo*) time. The data obtained in the characterisation of the antimicrobial supports and the sensory analysis were analysed by a one-way ANOVA. The least significance procedure (LSD) was used to test for differences between averages at the 5% significance level. Data were statistically processed by the Statgraphics Centurion XVI software.

3. Results and Discussion

3.1 Characterisation of MCM-41 microparticles

Table 1 shows the particle size ($d_{0.5}$) and ζ-potential values of the different silica particles. The bare and functionalised MCM-41 microparticles exhibited a $d_{0.5}$ between 0.6-1.9 µm. The non-modified particles and the supports functionalised with eugenol and vanillin showed particle size values around 1 µm, whereas the particles

funtionalised with carvacrol presented higher size values. The bare particles exhibited a negative ζ-potential value due to the presence of silanol moieties. After immobilisation however, the electrical charge of the functionalised microparticles changed to positive values due to the attachment of the bioactive compounds-alkoxysilane derivatives to the support surface.

Table 1. Particle size ($d_{0.5}$) and ζ-potential values of the bare MCM-41 microparticles and the microparticles functionalised with eugenol, carvacrol and vanillin. Mean values (n=3) ± SD.

Sample	$d_{0.5}$ (μm)	ζ-potential (mV)
Bare MCM-41	0.87 ± 0.01 [b]	-35.8 ± 3.9 [a]
Eugenol-MCM-41	0.62 ± 0.01 [a]	57.0 ± 2.2 [d]
Carvacrol-MCM-41	1.87 ± 0.31 [c]	43.2 ± 6.4 [b]
Vanillin-MCM-41	0.81 ± 0.04 [b]	52.6 ± 3.3 [c]

[a, b, c, d] *Different superscripts denote differences among the mesoporous silica supports*

The morphology of the bare and functionalised MCM-41 microparticles was assessed by FESEM. The particles showed hexagonal morphology with particle size in the microscale range. As seen in Figure 2, no modifications on the silica material surface were noticed after immobilising the MCM-41 microparticles with eugenol, carvacrol or vanillin. These results demonstrate that the immobilisation procedure does not alter the integrity of the mesoporous silica microparticles.

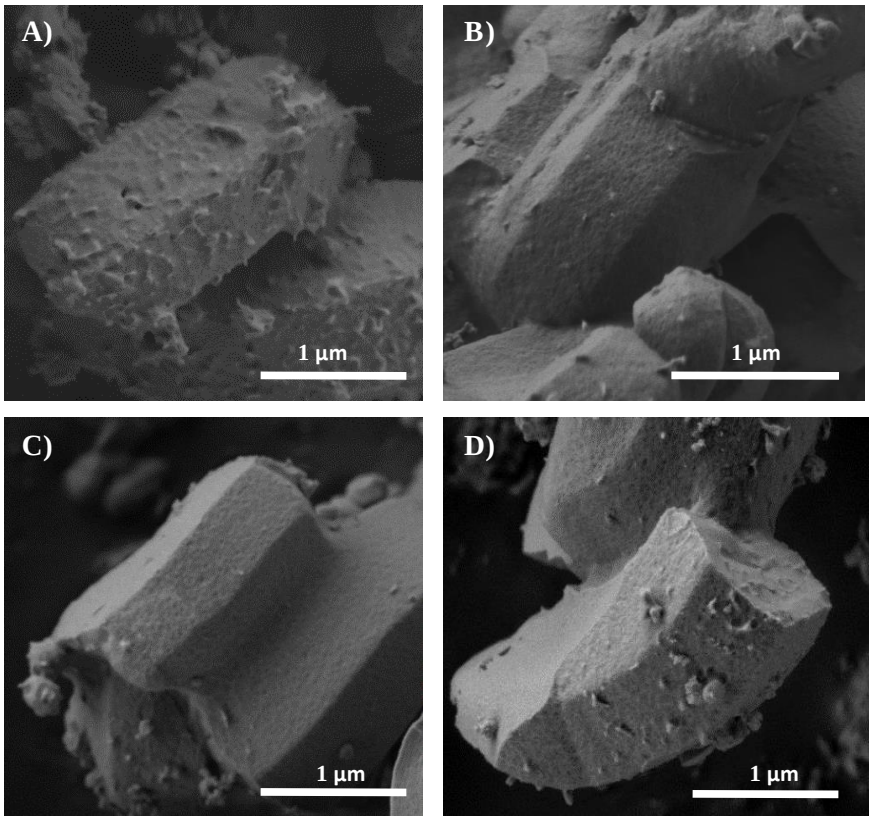

Figure 2. FESEM images of A) the bare MCM-41 microparticles, B) the eugenol-MCM- 41 microparticles, C) the carvacrol-MCM-41 microparticles and D) the vanillin-MCM-41 microparticles.

The N_2 adsorption-desorption isotherms allow us to calculate the structural properties of the starting MCM-41 material resulting in pore size of 2.81 nm, pore volume of 0.91 cm^3/g and specific surface area of 1016 m^2/g.

According to the thermogravimetric and elemental analyses, the amount of the bioactive compounds immobilised on the silica support was 121.7, 65.4 and 132.9 mg/g of SiO_2 for eugenol, carvacrol and vanillin, respectively.

3.2 Antimicrobial activity of the functionalised MCM-41 microparticles

3.2.1 In vitro antimicrobial activity of the immobilised eugenol, carvacrol and vanillin

The *E. coli* counts after treatment with immobilised bioactive compounds are shown in Figure 3. Non-growth inhibition was noted when the bare MCM-41 microparticles were used (Ribes et al., 2017). *E. coli* growth significantly reduced ($p<0.05$) as the concentration of the immobilised eugenol and vanillin increased. The use of 0.125 mg/mL of both immobilised compounds attained a *E. coli* reduction of 7-log cycles after 24 h, while microbial growth was completely inhibited at the end of the incubation period when 0.25 mg/mL of the immobilised eugenol and vanillin was used (Figure 3, A and C), which corresponds to the MIC and MBC values. Previous works have suggested that the great antimicrobial effectiveness of the immobilised molecules is due to the high surface concentration of the anchored bioactive compounds in the surface of particles that come into direct contact with the cell membrane (Botequim et al., 2012), which would activate membrane disruption mechanisms.

The immobilised carvacrol was unable to inhibit *E. coli* growth throughout the incubation period, regardless of the concentration employed (Figure 3B). Recently, Ruiz-Rico et al. (2017) evaluated the *in vitro* antimicrobial activity of thymol (an isomer of carvacrol) immobilised on different silica particles against *E. coli*, and these authors obtained a MBC of 0.2 mg/mL. The observed differences could be attributed to: i) the amount of bioactive compounds attached on the silica support; ii) the inoculum density; iii) the sensitivity of the microorganism to the action of each active compound or iv) the assay run to determine antimicrobial activity.

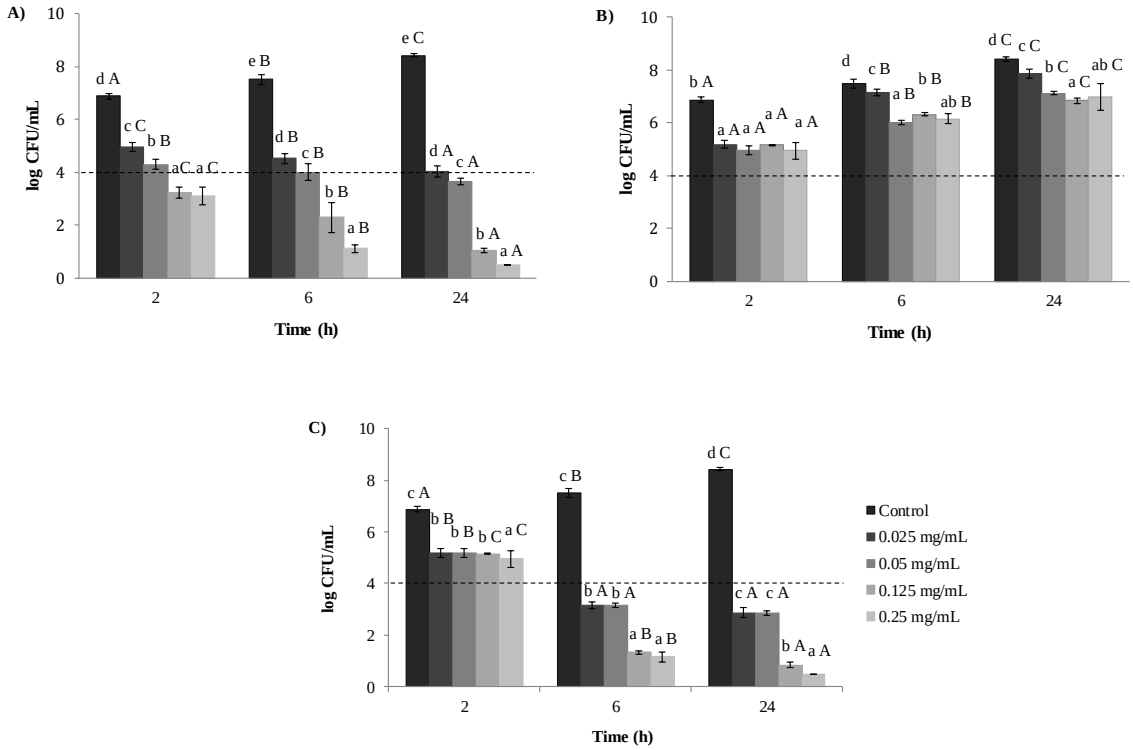

Figure 3. *E. coli* counts (log CFU/mL) after the treatment with different concentrations of the immobilised eugenol (A), carvacrol (B) and vanillin (C) at 37 °C for 2 h, 6 h and 24 h. Mean values (n=3) ± SD. Lowercase letters (a, b, c, d, e) indicate significant differences among the concentrations of the bioactive compounds ($p < 0.05$). Capital letters (A, B, C) indicate significant differences among incubation times ($p < 0.05$). The discontinuous horizontal line indicates the initial CFU/mL.

Figure 4 presents *Z. rouxii* counts after the 48-hour treatment with the immobilised bioactive compounds. Non-treated samples (positive control) presented a microbial growth of 7.17±0.08 log CFU/mL, whereas the samples incubated with the immobilised compounds showed the inhibition of the yeast. The immobilised eugenol and carvacrol presented remarkable antimicrobial activity, which was lower for vanillin. A strong correlation was found between the concentration of the bioactive agents and *Z. rouxii* growth inhibition. The use of 0.05 mg/mL of eugenol and carvacrol led to a reduction of

between 2 and 3 log-cycles at the end of the incubation period. Indeed the growth inhibition of yeast was achieved by using 0.125 and 0.25 mg/mL of the immobilised eugenol and carvacrol, respectively. The largest amount of vanillin used (0.25 mg/mL) did not inhibit *Z. rouxii* during the 48-hour incubation. Yeast growth reduced as the amount of functionalised vanillin increased. The use of 0.125 mg/mL and 0.25 mg/g of vanillin exhibited a reduction of 1 and 2 log-cycles of *Z. rouxii*, respectively.

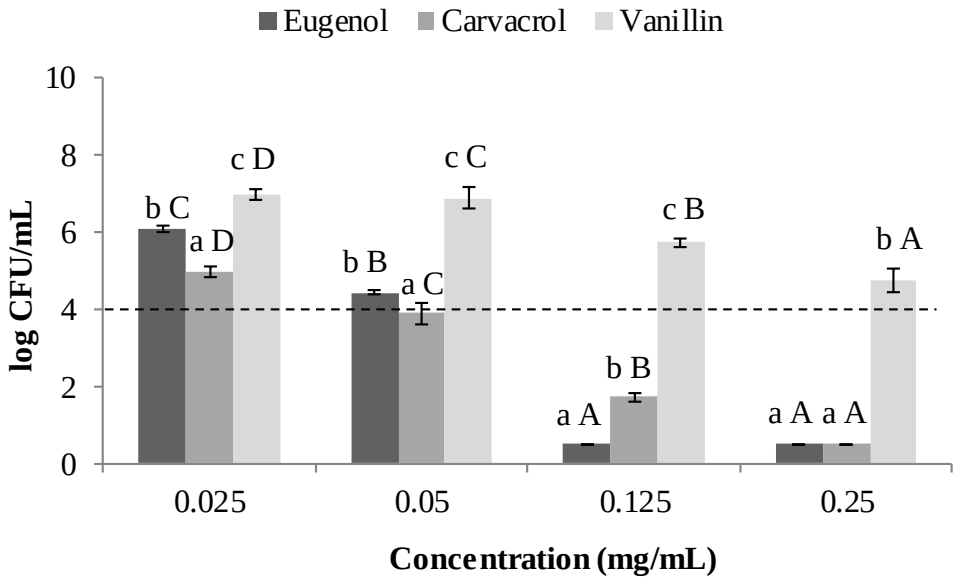

Figure 4. *Z. rouxii* counts (log CFU/mL) after the treatment with different concentrations of the immobilised eugenol, carvacrol and vanillin at 25 °C for 48 h. Mean values (n=3) ± SD. Lowercase letters (a, b, c) indicate significant differences among the bioactive compounds ($p < 0.05$). Capital letters (A, B, C) indicate significant differences among the concentrations of the bioactive compounds ($p < 0.05$). The discontinuous horizontal line designates the initial CFU/mL.

Fitzgerald, Stratford, & Narbad (2003) pointed out the sensitivity of *Z. rouxii* to free vanillin (MIC: 12 mM). Conversely, Rojo et al. (2015) employed a wide range of

free vanillin concentrations that reached only a 40% *Z. rouxii* growth reduction in a high sugar medium supplemented with the largest amount of pure vanillin (30 mM). The findings achieved herein can be explained by a sensitivity reduction of *Z. rouxii* to the vanillin treatments since the culture media conditions act as a protective environment against preservative efficiency.

3.2.2 In vivo antimicrobial activity of the immobilised eugenol, carvacrol and vanillin

Figure 5 shows the *E. coli* counts in the different fruit juices after incorporating eugenol and vanillin throughout refrigerated conditions. The growth inhibition of *E. coli* in fruit juices was dependent on the concentration of the functionalised microparticles and storage time. In apple juice, the use of 0.025 mg/mL of the immobilised eugenol led to a reduction of 2 log-cycles after a 24-hour refrigerated storage, whereas larger amounts of this solid caused bacteria inhibition after a 2-hour storage (MIC and MBC values: 0.05 mg/mL). In contrast, the inhibition of *E. coli* was observed only when the largest amount of vanillin was used (MIC and MBC: 0.25 mg/mL) at the end of the storage period. The *E. coli* growth inhibition in grape juice was noticed in all the samples treated with the immobilised eugenol, regardless of concentration and storage time. A vanillin concentration of 0.05 mg/mL resulted in a reduction of between 1 and 2 log-cycles, and higher concentrations produced the complete inhibition of *E. coli* after a 2-hour storage. In grape juice, the MIC and MBC of the vanillin-MCM-41 microparticles was 0.125 mg/mL.

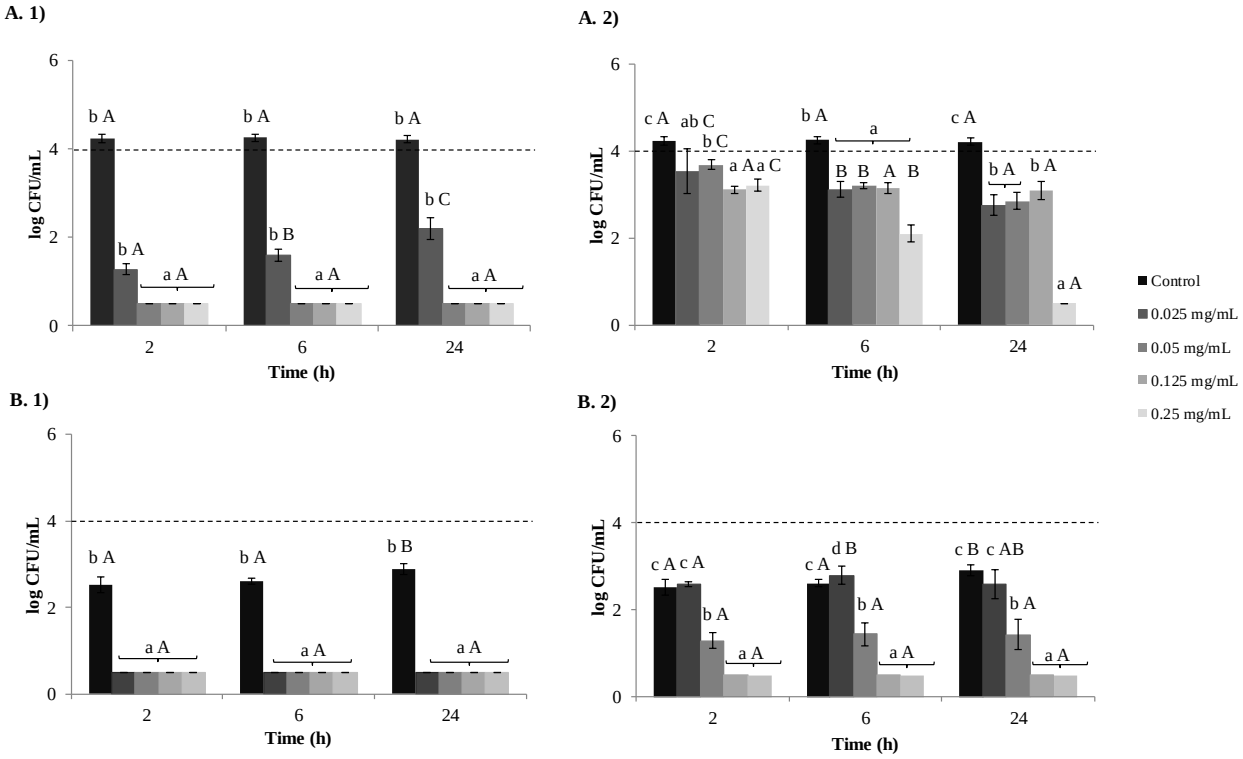

Figure 5. *E. coli* counts (log CFU/mL) in the inoculated apple (A) and grape (B) juices after incorporating different concentrations of the immobilised eugenol (A.1 and B.1) or vanillin (A.2 and B.2) at 8 °C for 2 h, 6 h and 24 h. Mean values (n=3) ± SD. Lowercase letters (a, b, c, d) indicate significant differences among the concentrations of the bioactive compounds ($p < 0.05$). Capital letters (A, B, C) indicate significant differences among storage times ($p < 0.05$). The black dotted line expresses the initial CFU/mL.

Despite the pH is a critical factor for microorganisms and one of the main preservatives for foods, *E. coli* has an acid adaptability mechanisms (Mazzota, 2001). For the apple juice samples, the pH and ° Brix values during the whole assay were 3.45±0.1 and 11.6±0.3, respectively. In the case of the grape juice, the pH of all the samples was 3.28±0.2 and the ° Brix values were 16.6±0.4. In the present study, the lower microbial count observed in control grape juice samples, which in turn implies an increase in the effectiveness of the immobilised compounds in this juice, could be

ascribed to the different nutritional composition and viscosity of the fruit juices employed (sugars, fibres and ascorbic acids among others). Nindo, Tang, Power, & Singh (2005) pointed out that the sugar composition and processing procedures of two different fruit juices could explain the differences observed in their rheological aspects like viscosity. Besides, according to Marques, Worcman-Barninka, Lannes, & Landgraf (2001) the viscosity parameter could be an inhibiting factor for the development of *E. coli* in unpasteurised juices and fruit pulps.

In addition to the effect of the food matrix, temperature storage is another factor to take into account on the antimicrobial properties of bioactive compounds. Different authors have observed that cold preservation improves *E. coli* survival under acidic conditions due to the reduced permeability of the cell membrane to protons and/or to reduced metabolic action (Baskaran, Amalaradjou, Hoagland, & Venkitanarayanan, 2010). This hypothesis is in accordance with the results obtained herein. However, the absence of assays at different temperature using the same matrix do not allow us to compare properly the results in terms of this factor. Therefore, further studies would be needed to establish the importance of the temperature parameter.

According to the results achieved in Section 3.2.1, the assays carried out with the fruit juices inoculated with *Z. rouxii* were conducted by using eugenol and carvacrol as antimicrobials (Figure 6). The incorporation of both immobilised compounds into the juices inhibited *Z. rouxii* growth in a concentration-dependent manner. The immobilised eugenol was more efficient in inhibiting yeast development in grape juice compared to carvacrol. Addition of 0.05 mg/mL of eugenol to this liquid food resulted in an approximate 3 log reduction of *Z. rouxii* compared to the control. Indeed the growth inhibition of the target strain was achieved with concentrations that went up to 0.05 mg/mL of eugenol, corresponding the MIC and MFC to 0.125 mg/mL of the

immobilised eugenol. The immobilised carvacrol exhibited greater effectiveness than eugenol in reducing fungi development in apple juice. Indeed the use of 0.05 mg/mL of the immobilised molecule caused a decrease of 2 log-cycles and the 0.125 mg/mL concentration avoided yeast development (MIC and MFC).

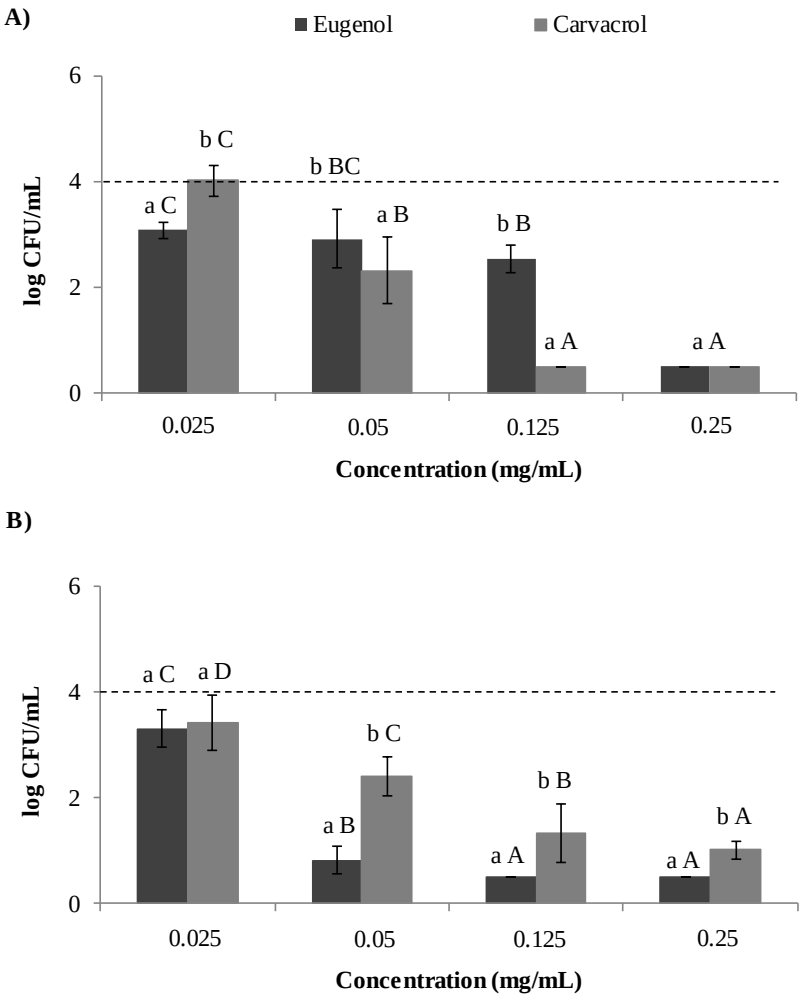

Figure 6. *Z. rouxii* counts (log CFU/mL) in the inoculated apple (A) and grape (B) juices after the treatment using different concentrations of the immobilised eugenol and carvacrol at 8 °C for 48 h. Mean values (n=3) ± SD. Lowercase letters (a, b) indicate significant differences among the bioactive agents ($p < 0.05$). Capital letters (A, B, C, D) indicate significant differences among the concentrations of the bioactive compounds ($p < 0.05$). The discontinuous horizontal line reflects the initial CFU/mL.

3.2.3 In vitro and in vivo antimicrobial activity of the immobilised bioactive compounds combinations

The *in vitro* FICi values of the dual combination (eugenol and vanillin or eugenol and carvacrol) against *E. coli* and *Z. rouxii* are presented in Table 2. The combination of the immobilised eugenol and vanillin or eugenol and carvacrol in culture media against *E. coli* and *Z. rouxii*, respectively, resulted in an additive or non-interactive effect. To the best of our knowledge, this is the first work to evaluate the synergistic activity of the immobilised bioactive compounds.

The synergistic activity of the immobilised bioactive compounds was also evaluated against *E. coli* and *Z. rouxii* in apple and grape juices. The mixture of the immobilised eugenol and vanillin had a non-interactive effect against *E. coli* in both fruit juices. Nevertheless, the immobilised eugenol and carvacrol combination against *Z. rouxii* displayed greater antimicrobial activity in the apple and grape juices. A synergistic activity was noticed between the antimicrobial supports in apple juice and an additive effect was observed by mixing these compounds in grape juice.

It is worth mentioning that multiple environmental factors (e.g., temperature, pH, a_w, physical matrix, etc.) determine the physico-chemical limits for yeasts' survival and growth, which led to the significant differences ($p<0.05$) observed between juices.

Table 2. Synergistic, additive or non-interactive interactions between the functionalised MCM-41 microparticles against *E. coli* and *Z. rouxii*.

In vitro test	***E. coli***		***Z. rouxii***	
	FIC	**FICi**	**FIC**	**FICi**
Eugenol-MCM-41	0.1	0.6	-	-
Vanillin-MCM-41	0.5		-	-
Eugenol-MCM-41	-	-	0.2	1.2
Carvacrol-MCM-41	-	-	1.0	
In vivo test	***E. coli***		***Z. rouxii***	
	FIC	**FICi**	**FIC**	**FICi**
Apple juice				
Eugenol-MCM-41	0.5	1.5	-	-
Vanillin-MCM-41	1.0		-	-
Eugenol-MCM-41	-	-	0.1	0.5
Carvacrol-MCM-41	-	-	0.4	
Grape juice				
Eugenol-MCM-41	1.0	1.5	-	-
Vanillin-MCM-41	0.5		-	-
Eugenol-MCM-41	-	-	0.2	0.7
Carvacrol-MCM-41	-	-	0.5	

3.3 Aroma reduction evaluation

The effectiveness of immobilisation process to reduce the effect of the bioactive compounds on the sensory attributes of foods were one of the most important aspects to be evaluated. In order to quantify the aroma reduction achieved by bioactive compounds immobilisation, the fruit juices were analysed by HS-SPME/GC-MS. The amount of each bioactive compound incorporated into the analysed juices (free or immobilised) corresponded to the MIC value.

Table 3 shows the reduction of the intensity response when free and immobilised eugenol, carvacrol and vanillin were compared. No immobilised vanillin was detected (100 % of aroma reduction) in apple and grape juices, which confirmed its covalent attachment, which hinders the release of the volatile product, and therefore, its aroma. Similarly, the immobilisation of eugenol and carvacrol allowed the typical aroma to be almost completely masked.

Table 3. Aroma reduction (%) for the immobilised bioactive compounds incorporated into fruit juices. Mean values (n=3) ± SD.

Juice	Bioactive compound	Aroma reduction (%)
Apple	Eugenol	98.6 ± 0.2
	Carvacrol	95.4 ± 0.1
	Vanillin	100.0 ± 0.0
Grape	Eugenol	98.2 ± 0.5
	Carvacrol	78.9 ± 5.3
	Vanillin	100.0 ± 0.0

3.4 Sensory evaluation

The sensory test was performed to evaluate the effect of the bioactive compounds on the sensory acceptance of fruit juices. The juices that contained combinations of the free and immobilised bioactive compounds and the control juices (no added antimicrobial compounds) were evaluated (Figure 7). The incorporation of the bioactive compounds (free or immobilised) led to significant differences ($p<0.05$) in general product acceptance and in the typical aroma, compared to the control juices. The general acceptance could be affected by the incorporation of the immobilised compounds given the presence of visible particles at the bottom of the samples.

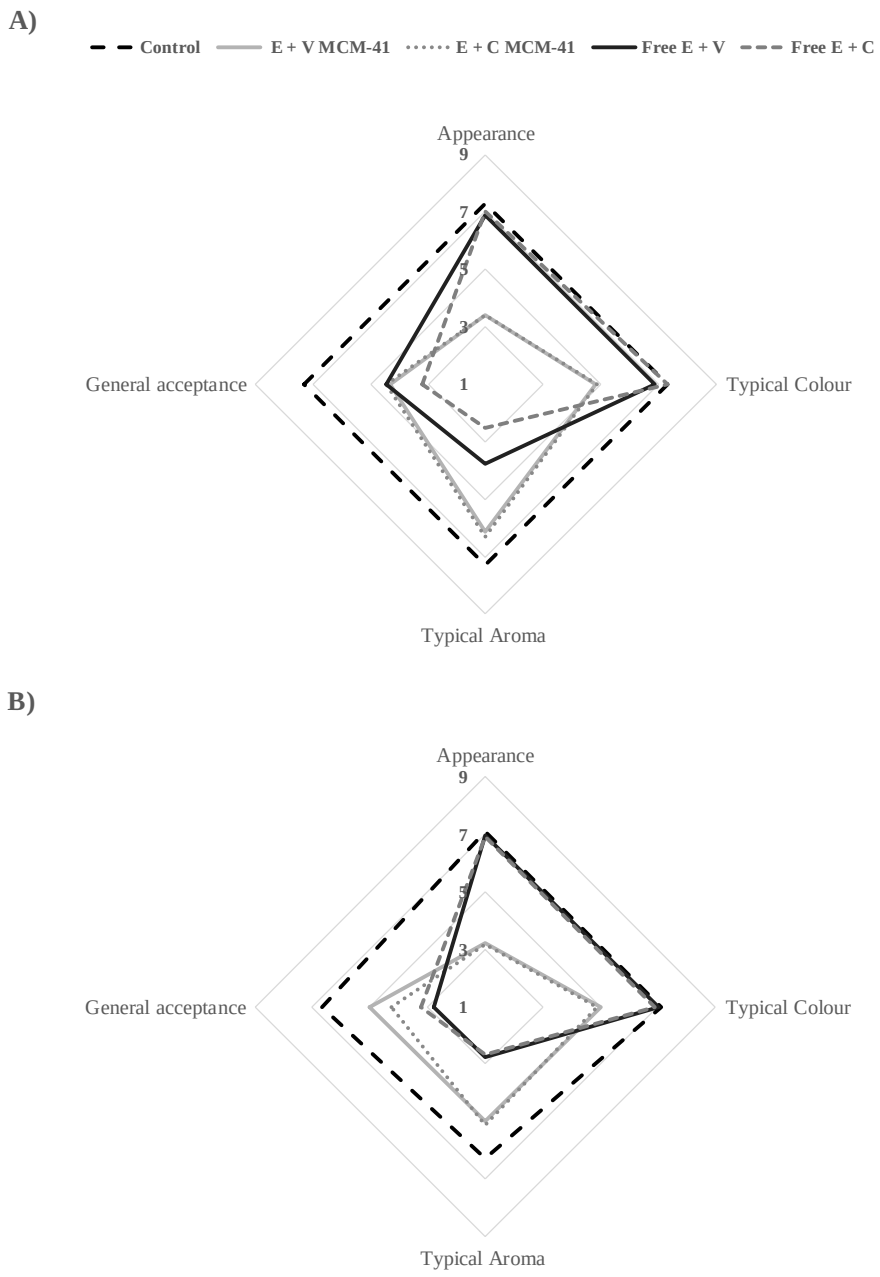

Figure 7. Average score of the different attributes evaluated in the apple juice (A) and grape juice (B), and the samples prepared with different combinations of the free and immobilised bioactive compounds. A 9-point hedonic scale where 0= very unpleasant and 9= very pleasant (n=30) (E: eugenol; V: Vanillin; C: carvacrol).

In the juice samples that contained bioactive compounds, the typical aroma differed from the control samples, mainly due to the huge impact of incorporating eugenol. Indeed despite the immobilisation not totally eliminated the huge impact of the bioactive compounds aroma on fruit juices, this system was able to attenuate their effect on food aroma. These results agree with those obtained by HS-SPME/GC-MS, where immobilisation was capable of reducing the aroma of eugenol and carvacrol, and completely eliminating the aroma of vanillin. Nevertheless, more studies should be carried out to make a positive correlation between the antimicrobial activity of these immobilised compounds and their impact on final food commodities (appearance, colour and general acceptance).

4. Conclusion

Immobilised bioactive compounds display remarkable *in vitro* antimicrobial activity against *E. coli* and *Z. rouxii*. Besides, the combined used of two of the immobilised bioactive compounds exhibits an additive or synergistic activity in the fruit juices. Immobilisation masks the characteristic undesirable aroma of the tested bioactive compounds in the food matrix. These facts offer the possibility of applying the immobilised bioactive compounds in two uses: (a) as food preservatives (if additives are present in the final food) or (b) as food processing aids (if particles are removed by decantation or centrifugation after microbial stabilisation).

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Conflict of interest

Authors declare that they have no conflicts of interest.

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