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Efficient reduction in vegetative cells and spores of *Bacillus subtilis* by essential oil components-coated silica filtering materials

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***B. subtilis* reduction by coated filters…**

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ABSTRACT: Inactivation of bacterial spores is a key objective for developing novel food preservation technologies. In this work, the removal properties of filtering materials based on silica microparticles functionalized with essential oil components (carvacrol, eugenol, thymol and vanillin) against *Bacillus subtilis*, a spore-forming bacterium, in two liquid matrices were investigated. The viability of vegetative cells and spores after treatment was also evaluated. The results exhibited marked removal effectiveness against *B. subtilis* vegetative cells and spores after filtration with the different silica supports coated with essential oil components (EOCs) in either sterile water or nutrient broth, with reductions of 3.2-4.9 log units 3.7-5.0 log units for vegetative cells and spores, respectively. The fluorescent viability images revealed the poor viability of the treated *B. subtilis* vegetative cells and spores due to damage to the cell envelope when coming into contact with the immobilized antimicrobials. The culture counts results revealed the great inhibitory capacity of the EOC-functionalized silica microparticles against *B. subtilis* vegetative cells and spores after a single filtration. Hence the present work suggests the feasibility of using EOC-functionalized supports as filtering aids to enhance the microbial quality of liquid matrices with spore-forming microorganisms.

Practical Application:

The developed antimicrobial-coated filters have shown remarkable removal properties against an important spore-forming bacterium in food industry. These filters may be used as a potential sterilization technique for preservation of different beverages alone or in combination with other mild-thermal or non-thermal techniques.

1 Introduction

Bacterial spores are of great concern to the food industry owing to their high resistance to physico-chemical preservation treatments (Wuytack & Michiels, 2001), which can result in spore germination and, therefore, food spoilage or potential foodborne diseases (Fan et al., 2019). Inactivation of spores has traditionally been carried out by heat treatments, but these processes are detrimental for food quality (Chemat, Zill-E-Huma, & Khan, 2011). Nevertheless, the rising consumers' demand for high quality fresh-like foods, with an extended shelf-life, has forced the food industry to look for alternative processing methodologies (Koutchma, 2009; Soni, Oey, Silcock, & Bremer, 2016).

Different non-thermal technologies such as high pressure processing, pulsed electric fields, irradiation, pulsed light treatment, filtration and natural antimicrobial agents have been recently used to inactivate bacterial spores (Artíguez & Martínez De Marañón, 2015; Lawrence & Palombo, 2009; Milani & Silva, 2017; Reverter-Carrión et al., 2018; Zhao et al., 2015). Among them, filtration is a non-thermal process widely used for the clarification, concentration and microbial stabilization of beverages. Filtering beds based on sand, silica or cellulose of 1-1000 µm are used for the removal of organic matter and microorganisms, but they do not possess an adequate efficacy for microbial elimination due to the high size of the filtering particles (Devi, Alemayehu, Singh, Kumar, & Mengistie, 2008). On the other hand, microfiltration with very small pore size (0.2-0.45 µm) has been used as sterilization treatment for juice, beer or wine industries given its retention capability against microorganisms. Nevertheless, the main limitation of the use of filtering materials with a very low pore size is clearance of beverages by retention of color, flavor and nutritional components (Zhao et al., 2015).

The use of naturally-occurring antimicrobial compounds to replace, or to be used in combination with other preservation methods to inactive spores, has also been reported. Essential oils and their active components (EOCs) display sporicidal activity with damage to the spore coat, which results in damage to the spore structure and the release of nucleic acids and proteins (Cai et al., 2019). They also inhibit germination through the interruption of the response to the nutrient receptors involved in the cascade of changes from a dormant form to a vegetative cell (Lawrence & Palombo, 2009). Despite the potential efficacy of EOC in food matrices as sporicidal agents (Bevilacqua, Corbo, & Sinigaglia, 2008; Valero & Giner, 2006; Voundi et al., 2015), their use in the food industry is limited given their strong sensory properties, poor solubility and instability (Hyldgaard, Mygind, & Meyer, 2012).

As an alternative to direct administration, antimicrobial agents can be immobilized on the surface of different substrates to develop materials with additional functionalities, while maintaining their bulk material properties. Following this approach, EOCs have been immobilized on silica microparticles to develop antimicrobial supports, preserving the bioactive properties of grafted compounds (Ribes, Ruiz-Rico, Pérez-Esteve, Fuentes, & Barat, 2019). Recently, the removal capability of EOC-functionalized silica microparticles used as filtering materials has been reported against vegetative cells of different spoilage and pathogen microorganisms (Peña-Gómez, Ruiz-Rico, Pérez-Esteve, Fernández-Segovia, & Barat, 2019b; Peña-Gómez, Ruiz-Rico, Fernández-Segovia, & Barat, 2019a; Peña-Gómez, Ruiz-Rico, Pérez-Esteve, Fernández-Segovia, & Barat, 2020). However, to date no research is available evaluating the effect of these antimicrobial filtering supports on bacterial spores. Hence, the aim of this work was to establish the removal properties of the filtering materials formed from biocompatible materials (silica gel as a support and EOCs as

immobilized antimicrobial agents) against the relevant spore-forming bacterium *Bacillus subtilis* in different matrices. For that, the removal properties of silica supports of different particle size (25 µm and 200 µm) covalently coated with essential oil components (carvacrol, eugenol, thymol and vanillin) were investigated against vegetative cells and spores of *Bacillus subtilis* in two liquid matrices (water and nutrient broth). Liquid matrices were filtered through filtering aids and the microbial load was determined by plate count and the viability of vegetative cells and spores after treatment was evaluated by cell culture count and fluorescent viability staining techniques.

2 Materials and Methods

2.1 Reagents

Carvacrol (≥99% w/w), eugenol (99% w/w), thymol (≥99% w/w), amorphous silica microparticles (mean sizes of 25 µm and 200 µm), (3-aminopropyl)triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butanone and sodium borohydride were provided by Sigma-Aldrich (Madrid, Spain). Vanillin (>99% w/w) was supplied by Ernesto Ventós S.A. (Barcelona, Spain). Acetonitrile, HCl, NaCl, KCl, Na_2HPO_4, KH_2PO_4, $MgSO_4$, $MnSO_4$, KOH and H_2SO_4 were acquired from Scharlab (Barcelona, Spain).

2.2 Synthesis of EOC-functionalized materials

Silica microparticles of mean particle sizes of 25 µm and 200 µm were used as inorganic supports for the immobilization of naturally-occurring antimicrobial compounds (carvacrol, eugenol, thymol or vanillin) by surface silanization, which resulted in EOC-functionalized materials (see Figure S1 for details of the synthesis procedure). The use of organosilane coupling agents allowed the

immobilization of EOCs by covalent bond with amino functional group of the organosilane APTES. The synthesis of EOC-functionalized materials was performed following a four-step reaction. Firstly, the synthesis of aldehyde derivatives was performed to add a second reactive moiety capable of reacting with APTES (Chen, Shi, Neoh, & Kang, 2009). With carvacrol and thymol, this was carried out by direct formylation using paraformaldehyde. For eugenol, the aldehyde derivatives were obtained by the Reimer-Tiemann reaction. As a second step, the EOC-alkoxysilane derivatives were synthesized by a reaction of APTES with the aldehydes of carvacrol, eugenol and thymol, as well as pure vanillin by Schiff base reaction via imine bonding. Next these derivatives were grafted to the supports' surface by reaction of the EOC-alkoxysilane derivatives with the silanol groups (Si–OH) present on the surface of silica microparticles in form of siloxane bonds. Finally, the imine bond formed between the aldehyde group of each EOC and the amino group of organosilane was reduced to amine bond to stabilize the chemical bond of the immobilized EOC. The synthesis protocol is fully described in García-Ríos, Ruiz-Rico, Guillamón, Pérez-Esteve, and Barat (2018).

2.3 EOC-functionalized materials characterization

The bare and EOC-functionalized silica microparticles were characterized by standard techniques to establish their particle size, surface charge and degree of functionalization. Particle size was determined in distilled water using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The Mie theory was applied by using a refractive index of 1.45 and an absorption index of 0.01. The results were expressed as $d_{0.5}$, which corresponds to the median of the samples' particle size distribution. The zeta potential analysis was run in a Zetasizer Nano ZS

(Malvern Instruments, Worcestershire, UK). Samples were dispersed with distilled water (1 mg/mL) and sonicated before being analyzed to prevent the agglomeration of silica microparticles. The electrophoretic mobility measurements were transformed into zeta potential values based on the Smoluchowsky mathematical model. The degree of functionalization was determined by thermogravimetric analysis (TGA), which were performed on a TGA/SDTA 851e balance (Mettler Toledo, Columbus, USA) from 25ºC to 1,000ºC at a heating rate of 10ºC/min in an oxidant atmosphere (air, 80 mL/min). The results were expressed as α (g of organic matter/g solid). The bulk density of the silica supports was determined by pouring around 20 g of support into a 100-mL measuring cylinder and tapping 10 times on a flat wooden platform. The mass of particles and the volume occupied by the samples were recorded. Bulk density was expressed as the mass/volume ratio (g/cm^3). All the analyses were done in triplicate.

The data acquired from the different characterization techniques were used to calculate the number of particles/g of solid, EOC content/g of particle and the EOC density on the particles' surface assuming that particles morphology can be simplified to a sphere. The number of particles per gram of solid and the surface area per gram of solid were calculated according to Eq. 1 and 2. The content of the organic matter (determined by the TGA) and the mean average particle size (determined by laser diffraction) values were used to estimate the number of EOC molecules/g solid (Eq. 3). Finally, the density of EOCs on the particles' surface was calculated taking into account the surface area per gram of solid (Eq. 4).

Number of particles/g solid = 1 / ((particle's volume (cm^3) x density (g/cm^3)) (Eq. 1)

Surface area (nm^2/g solid) = number of particles (particles/g solid) x particles' area (nm^2) (Eq. 2)

149 EOC molecules/g solid = (α (g/g solid) / molecular weight (g/mol)) x 6.023 x 10^{23} (Eq. 3)

150 EOC density (molec/nm^2) = EOC molecules (molec/g solid) / surface area (nm^2/g solid) (Eq. 4)

151

152 **2.4 Reference strain and growth conditions**

153 *Bacillus subtilis* (CECT 461) was obtained from the Spanish Type Culture Collection (CECT, Burjassot,
154 Spain). The microorganism was grown on nutrient agar (NA) plates (Scharlab, Barcelona, Spain)
155 and incubated at 37ºC for 24 hr, as recommended by the provider.

156

157 **2.5 Bacterial inoculum preparation**

158 To prepare the bacterial inoculum, the *B. subtilis* strain was grown as described above. Afterwards,
159 colonies were transferred to a test tube containing 10 mL of phosphate-buffered saline (PBS 1X, 8
160 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4) to obtain an inoculum with a microbial
161 density of $1x10^6$ cells/mL. Vegetative cells were counted by fluorescence microscopy.

162

163 **2.6 Spore suspension preparation**

164 To prepare the spore suspension, *B. subtilis* was grown on NA plates supplemented with 5 mg/L of
165 $MnSO_4$ (NA-Mn) and incubated at 37ºC for 24 hr, as performed by Pinto, Santos, Fidalgo, Delgadillo,
166 and Saraiva (2018) with minor modifications. Briefly, after incubation, the biomass was scraped
167 from the NA surface and washed with 10 mL of sterile distilled water. The resulting suspension was
168 incubated at 80ºC for 15 min to inactivate vegetative cells. When cooling, the suspension was
169 centrifuged (10,000 rpm for 10 min at 5ºC), the supernatant was decanted, and the spore pellet
170 was washed with cold sterile distilled water. This step was done in duplicate. Next the suspension

was incubated at 37ºC for 60 min in the presence of lysozyme (100 µg/mL) to achieve peptidoglycan cell wall breakdown to facilitate the release of spores (Pinho, Nunes, Lobo-da-Cunha, & Almeida, 2015). Afterwards, the mixture was centrifuged as previously described. Finally, the *B. subtilis* spore suspension was washed twice with sterile distilled water. To confirm the presence of spores, malachite green staining of the *B. subtilis* stock suspension was performed by a Motic BA310E trinocular microscope. The spore stock suspension, at a final concentration of approximately $1x10^6$ cells/mL, was stored at 4ºC for 1 month until further use (Wells-Bennik et al., 2019).

2.7 Removal capability of the EOC-functionalized materials against a spore-forming microorganism

The removal capability of the EOC-functionalized supports against *B. subtilis* was evaluated with all the developed filtering materials, which means particles of 25 µm and 200 µm were functionalized with carvacrol, eugenol, thymol or vanillin. For that purpose, two different matrices (water and nutrient broth) were inoculated with the target microorganisms to evaluate the effect of nutritional conditions on bacterial viability and germination.

The removal capability assays were performed by filtering 100 mL of sterile distilled water (SW) inoculated with *B. subtilis* vegetative cells or spores at a final concentration of $1x10^3$ cells/mL through a bed composed of the functionalized microparticles (layer thickness of 0.5 cm), cellulose paper and a cellulose membrane filter (0.45 µm) that retained the microbial cells. After filtration, the cellulose membrane filter was transferred to NA plates and incubated at 37ºC for 24 hr. These analyses were also conducted by using sterile nutrient broth (NB) and NB supplemented with 5

193 mg/L of $MnSO_4$ (NB-Mn) for vegetative cells and spores, respectively, to study the removal
194 capability of the EOC-functionalized filters in a matrix that favored bacteria survival and spore
195 germination.
196 The non-filtered samples (the reference sample filtered only with the 0.45 µm cellulose membrane
197 filter, but not with filtering supports) and the samples filtered through a bed of bare silica
198 microparticles were included in the assays as the control samples. The filtration assays were
199 performed using a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt,
200 Germany). The results were expressed as the decimal logarithm variation (log (N/N_0)) obtained by
201 the difference between the microbial load after filtration (N) and the initial microbial load (N_0). To
202 calculate the decimal logarithm variation, the microbial counts present in 100 mL of medium were
203 considered (e.g., 5 log/100 mL as the initial microbial load). Tests were conducted in triplicate.
204
205 **2.8 Bacterial cell and spore viability determination**
206 The influence of filtration through the EOC-functionalized silica microparticles on bacterial and
207 spore viability was studied by cell culture counts and fluorescent viability staining techniques.
208 The viability assays were run on: i) the vegetative cells/spores retained on the cellulose membrane
209 filter; ii) the vegetative cells/spores retained on the bed of particles; iii) the vegetative cells/spores
210 present in the liquid medium (SW or NB/NB-Mn) recovered after filtration. In all cases, 100 mL of
211 SW or NB/NB-Mn were inoculated with $1x10^5$ cells/mL of *B. subtilis* (vegetative cells or spores
212 suspension) and filtered through the microparticles' bed with a thickness of 0.5 cm.
213 The EOC-functionalized supports used for performing these assays were selected according to the
214 results obtained with the removal capability tests. Thus the most effective systems used were the

25 µm and 200 µm-supports functionalized with thymol and vanillin. The non-filtered samples (reference sample) and the liquid media filtered through a bed of bare silica microparticles were included in the assays as the control samples. Tests were done in triplicate. A scheme of the experimental procedure herein followed is shown in Figure 1.

2.8.1 Viability assessment of the cells/spores retained on the cellulose membrane filter

After filtration, the cellulose membrane filter (0.45 µm), on which the microorganism was retained, was scraped in 1 mL of SW or NB/NB-Mn to assess the viability of the biomass retained on these filters by a fluorescent staining technique (LIVE/DEAD® BacLight Cell Viability Kit; Invitrogen, ThermoFisher Scientific, UK). For this assay, 0.4 µL of a mixture of SYTO 9 and propidium iodide (PI) dyes (ratio 1:1) were incorporated into 250 µL of the microbial suspension. Subsequently, the preparation was mixed and incubated at room temperature for 10 min and contact with light was avoided. Then 5 µL of the stained cells were laid on poly-*L*-lysine-covered slides (Sigma-Aldrich, Madrid, Spain) and sealed with a coverslip. Finally, slides were visualized under a Motic BA310E trinocular microscope equipped with an Epi-Led module, MB barrier filter and a Moticam 3+ camera.

The same methodology was used to establish the viability of both vegetative cells and spores present in the recovered liquid after filtration. Green-fluorescing SYTO 9 penetrates membrane and cortex of viable or dead spores, whereas red-fluorescing PI penetrates exclusively those spores with damaged inner membrane and cortex (Berney, Hammes, Bosshard, Weilenmann, & Egli, 2007; Park, Yang, Choi, & Kim, 2017) and, therefore, the viability staining with SYTO9/PI is a suitable

technique in determining structural damage of spores and can be used as an indicator of spores' viability after filtration (Wang et al., 2020).

2.8.2 Viability assessment of the cells/spores retained on the bed of particles

Viability assays were also conducted on the cells/spores retained on the bed of particles. After filtration, the bed of particles was transferred to a stomacher bag and resuspended in 10 mL of SW or NB/NB-Mn to be incubated at 37ºC for 24 hr. After the incubation of the biomass present on the bed of particles, cells were observed by the above-described fluorescent staining technique.

2.8.3 Viability assessment of the cells/spores present in the liquid medium recovered after filtration

For this assay, the SW or NB/NB-Mn inoculated with the *B. subtilis* vegetative cells or spores were filtered through the bed of EOC-functionalized supports without using the 0.45 µm-cellulose filter, which resulted in non-retention of the microbial cells or spores in the membrane. The filtration assays were performed using a stainless steel manifold connected to a sterile Erlenmeyer flask to collect the filtered media. To determine the viability of the vegetative cells/spores present in the recovered liquid medium after filtration, the medium was centrifuged (10,000 rpm for 10 min at 5ºC) and the obtained pellet was resuspended in 1 mL of PBS. Cultivable cell numbers were established by plating 100 µL of each microbial and spore suspension on NA plates. Next plates were incubated at 37ºC for 24 hr and the results were expressed as log CFU/mL. To assess the viability of the vegetative cells/spores present in the recovered liquid medium, the fluorescent staining technique was followed, as explained earlier.

2.9 Statistical analysis

The results obtained from evaluating the removal capability of the EOC-functionalized silica microparticles and their antimicrobial/sporicidal activity were determined by a multifactor analysis of variance (multifactor ANOVA) to evaluate the influence of the three factors, functionalization, media and particle size used, as well as their interactions. The data acquired from the characterization of filtering supports was analyzed by a one-way ANOVA test. The least significance procedure (LSD) was followed to test for differences between averages at the 5% significance level. The results were statistically processed by the Statgraphics Centurion XVI Software.

3 Results and Discussion

3.1 EOC-functionalized materials characterization

Table S1 shows the particle size distribution expressed as $d_{0.5}$ of the bare and EOC-functionalized supports dispersed in distilled water. The 25 µm-supports presented $d_{0.5}$ values within the 21.7-25.5 µm range, which corresponded to the mean particle size established by the manufacturer. For the bigger supports, particle size was more heterogeneous, with $d_{0.5}$ values between 160.1 µm and 185.0 µm, which were lower than the values on the specification sheet. Grafting the EOCs on the surface of particles significantly affected ($p<0.05$) the support's particle size distribution, but the decreases or increases in particle size in suspension were slight.

The surface charge of the particles suspended in distilled water was determined by zeta potential. Figure S2 displays the zeta potential values of the non-functionalized and EOC-functionalized supports. The bare supports presented negative zeta potential values, which are related to the

deprotonated silanol groups of particles' surface. In contrast, the EOC-functionalized particles showed positive zeta potential values because of grafting the EOC-alkoxysilane derivatives on particles' surface. The positively charged EOC-functionalized supports could show electrostatic attraction with the negatively charged bacterial surface to favor supports' retention capability (Peña-Gómez et al., 2019b).

Table 1 shows the degree of functionalization of the EOC-functionalized materials according to the amount of organic matter (α) grafted onto particles and the estimated density of EOCs on the particles' surface. The supports functionalized with carvacrol, eugenol and thymol presented α values between 0.0676 and 0.01003 g/g solid, whereas the organic matter content was higher for the vanillin-functionalized supports with α values of ca. 0.16 g/g solid. These values are in accordance with the degree of functionalization reported in previous works (Peña-Gómez et al., 2019b; Ruiz-Rico, Moreno, & Barat, 2020). The EOC density on the particles' surface depended on organic matter content (α) and mean particle size of the particles, being the vanillin-functionalized supports the materials with the highest coating density.

3.2 Removal capability of the EOC-functionalized materials against a spore-forming microorganism

The removal capability of the EOC-functionalized materials was evaluated on *B. subtilis*, a strain of great concern to the food industry due to its ability to form highly resistant spores and used as surrogated endospores of the pathogenic *B. cereus* that produce toxins resulting in food poisoning (Pinto et al., 2018; Reineke et al., 2013). The removal properties were studied by the two spore-

301 forming microorganism forms (vegetative cells and spores) to assess the impact of the bacterial
302 physiological state on the effectiveness of the EOC-functionalized supports.
303
304 **3.2.1 Removal capability of the EOC-functionalized materials on vegetative cells**
305 Figure 2 shows the removal capability results of the bare and EOC-functionalized materials against
306 the *B. subtilis* vegetative cells inoculated in SW and NB. As this figure depicts, the use of bare silica
307 particles as filtering materials did not reduce the initial microbial load of *B. subtilis* (10^5 cells/100
308 mL) in spite of the media and mean particle size used. These results fall in line with those observed
309 by Onnis-Hayden, Hsu, Klibanov, and Gu (2011) when using untreated sand filters, mainly made of
310 silicate materials and silicate rock granular particles, for water disinfection. Nevertheless, marked
311 removal effectiveness against *B. subtilis* cells was noticed after the filtration with EOC-
312 functionalized supports in either SW or NB, and reductions fell within the range of 3.2-4.9 log units.
313 The statistical analysis of the results revealed that the immobilized compound and media, and the
314 functionalization-particle size and functionalization-media-particle size interactions, significantly
315 ($p<0.05$) influenced the removal of *B. subtilis* vegetative cells (Table S2).
316 According to the immobilized EOC, the vanillin-functionalized supports exhibited the greatest
317 removal capability, regardless of the media and particle size assessed. On the contrary, the lowest
318 removal capability was observed after filtering the inoculated NB through a bed of carvacrol-
319 functionalized supports with a mean particle size of 200 μm (reduction of 3.2±0.4 log units).
320 Previous works have suggested that the marked antimicrobial effectiveness of the immobilized
321 molecules is due to the high surface concentration of the anchored bioactive components on the
322 surface of the particles that come into direct contact with the cell membrane and activate

membrane disruption mechanisms (Botequim et al., 2012). Hence the differences observed in the removal capability of the EOC-functionalized supports could be attributed to the higher organic matter (α) content grafted and coating density for the vanillin-functionalized silica microparticles (*vide supra*).

No clear trend was observed for the used medium, but this factor had a significant impact on the reduction of the spore-forming bacterium. The removal properties of supports were expected to be higher in a simple medium, like water, than in broth due to the presence of peptides or sugars that can provide a protective effect for the microorganism (Ahn et al., 2015). However, this effect may be very slight as cells were not incubated in the media for a long time, but only for the time required to filter the sample. Therefore, the influence of the media's nutritional conditions would be limited (Pinto et al., 2018).

3.2.2 Removal capability of the EOC-functionalized materials on spores

Figure 3 presents the removal capability results of the EOC-functionalized supports against the *B. subtilis* spores inoculated in SW or NB. The filtration of the samples inoculated with *B. subtilis* spores through a bed of a bare 25 µm-support led to reductions of up to 3.0 log units. This reduction is produced by the retention of part of the spores load on the bed support, but the viability of the retained microbial cells may not be compromised by this effect (*vide infra*).

The EOC-functionalized supports exhibited significant removal properties on the target spores with reductions within the 3.7-5.0 log units range, and were able to completely eliminate the inoculated spores in some cases. The ANOVA analysis showed that the factors that significantly ($p<0.05$) influenced the removal properties of *B. subtilis* spores were the functionalization and particle size

used, as well as the functionalization-media and functionalization-particle size interactions (Table S2). The surface-coating boosts the retention of the spores in the filtering materials. Previous studies have shown that the capability of single-walled carbon nanotubes to remove spores was enhanced by different surface modifications, including titanium dioxide coating (Krishna et al., 2005) and saccharide functionalization (Wang et al., 2006).

According to the immobilized EOC, good spore removal capability was noticed with the thymol-functionalized filters in either SW or NB, and despite the mean particle size used (reduction of *ca.* 4.5 log units). A similar trend was observed when filtering the SW and NB-Mn inoculated samples through the carvacrol and vanillin-functionalized supports. Both the EOC-functionalized microparticles were able to completely remove *B. subtilis* spores from the NB-Mn medium when the 25 µm-filtering materials were used. The 25 µm-eugenol supports were also capable to remove the total spore load present in the SW sample after filtration.

Previous studies have evaluated the EOC impact on the viability of vegetative cells and spores, as well as their influence on spore germination. Lawrence and Palombo (2009) pointed out that *B. subtilis* spores treated with different essential oils looked withered and deflated with pronounced ridges. These authors associated the weakening spore membrane structure with the collapsed morphology noted after performing spore treatment with several essential oils. Moreover, Cortezzo, Setlow and Setlow (2004) established that disturbing the action of nutrient receptors involved in the cascade of changes that lead to germination was the mechanism by which essential oils could both reversibly and irreversibly inhibit spore germination. These findings suggest that the immobilized EOC that comes into direct contact with spores during filtration can bring about

366 many damages to their coats and promote loss of intracellular material and interrupt posterior
367 germination (Lawrence & Palombo, 2009).
368 By considering the removal results, it can be stated that EOC-functionalized supports present a
369 remarkable removal capability against spores (reduction of *ca.* 4.7 log units), and their use as
370 filtering aids could be a feasible preservation alternative against spore-forming microorganisms.
371 The results obtained in this work are in the same range as other non-thermal technologies. The
372 use of other filtration systems, like microfiltration, has been proved effective in removing spores
373 from beverages. Zhao et al. (2015) investigated the combined effect of microfiltration with ceramic
374 membranes (pore sizes of 0.8 and 1.4 µm) and UV treatment for the reduction of *Alicyclobacillus*
375 *acidoterrestris* from apple cider. Microfiltration with a 0.8-µm-pore-size membrane was capable of
376 reducing the vegetative cells and spores of *A. acidoterrestris* by 5 log units, while a 1.4 µm-
377 membrane achieved a reduction of 4.8 log units. However, the use of this filtering material with a
378 small pore size resulted in pore blocking and cake layer formation on the membrane surface, and
379 a chemical cleaning cycle (water rinses and alkaline cleaning) was required after each experiment.
380 Other non-thermal treatments have been proposed in the food industry to inactivate spores, such
381 as high pressure, pulsed light or UV, which are often combined with heat treatment or other non-
382 thermal technologies (Artíguez & Martínez De Marañón, 2015; Espejo, Hernández-Herrero, &
383 Trujillo, 2014; Reverter-Carrión et al., 2018).
384
385 **3.3 Bacterial cell and spore viability determination**
386 Considering the removal capability results, the thymol and vanillin-functionalized supports were
387 selected as materials for the viability assays for their excellent removal efficiency. The impact of

388 filtration on bacterial and spore viability was evaluated by different techniques, such as cell culture
389 counts and fluorescent viability staining, by using a higher microbial load ($1x10^5$ cells/mL of *B.*
390 *subtilis* vegetative cells or spores) to better visualize cells by microscopic techniques.
391 Firstly, the antimicrobial properties of the EOC-functionalized supports were established by
392 determining the viability of the *B. subtilis* vegetative cells and spores retained on the cellulose
393 membrane filter by a fluorescent staining technique (SYTO 9/IP). Figure 4 presents the fluorescence
394 microscopy images of the *B. subtilis* cells and spores of the inoculated SW and NB/NB-Mn samples
395 that were filtered, or not, through the bare and EOC-functionalized supports. As observed, the non-
396 filtered samples and samples filtered through the bed of bare silica microparticles (25 µm and 200
397 µm) exhibited green-stained cells and spores, which confirm their viability (Cronin & Wilkinson,
398 2007; Peña-Gómez et al., 2019b). Nevertheless, after filtering the inoculated samples through the
399 bed of EOC-functionalized supports, non-viable cells and spores (red-stained cells/spores), or a few
400 viable cells and spores (green-stained cells/spores), were detected.
401 Secondly, fluorescent viability staining was also used to determine the viability of the cell/spores
402 retained on the bed of bare and EOC-functionalized supports after incubation with the
403 corresponding medium at 37ºC for 24 hr (Figure 5). This figure shows the presence of viable cells
404 (green-stained) in the suspension and/or adsorbed on the silica microparticles surface after
405 filtering the samples through the bed of non-functionalized supports (25 µm and 200 µm). This
406 outcome suggests the partial retention of *B. subtilis* cells in bed particles during filtration.
407 Furthermore, the maintenance of the viability of the cells retained on the bed of particles after
408 incubation confirms the absence of antimicrobial activity by the bare supports (Onnis-Hayden, Hsu,
409 Klibanov, & Gu, 2011; Xiong et al., 2018). On the contrary, when filtering the inoculated SW and

410 NB samples through the bed of thymol and vanillin-functionalized supports, scarcely any viable
411 cells were detected, and the presence of non-viable cells predominated as a result of contact with
412 the EOC-functionalized support during filtration and incubation.
413 Regarding the *B. subtilis* spores (Figure 5), the samples filtered through a bed of bare silica
414 microparticles exhibited green-stained spores in suspension. This confirms the partial retention of
415 the *B. subtilis* spores in the bed of non-functionalized and EOC-functionalized particles during
416 filtration as previously observed in Figure 3. The use of EOC-functionalized supports resulted in the
417 presence of a low number of viable cells retained in the particles' bed after filtration. This can be
418 related to the presence of subpopulations of activated and dormant spores in the liquid matrices
419 (Pinho et al., 2015). After filtration, the activated subpopulation retained in the support bed can
420 be more sensitive to the antimicrobials effect and lose their viability, whereas the dormant spores
421 can remain viable, being present in the bed of particles.
422 To evaluate the viability of *B. subtilis* vegetative cells and spores, plate counts were conducted in
423 the recovery medium after filtration without using the 0.45-µm cellulose filter, which resulted in
424 the non-retention of microbial cells in the membrane. Figure 6 illustrates the *B. subtilis* cultivable
425 cells number in the recovery medium obtained after filtering the inoculated samples through the
426 bed of bare and EOC-functionalized supports. No cultivability inhibition was observed after
427 filtration through the bare supports in accordance with the initial microbial load (7.0 log CFU/100
428 mL). Vegetative cells and spores percolated through the bed of particles, and only partial retention
429 of vegetative cells was observed for the 25 µm-bare silica microparticles (NB) owing to the physical
430 adsorption that came about during filtration (Figure 3).

Conversely, the results revealed that the EOC-functionalized supports displayed great antimicrobial and sporicidal activity with reduction values of between 2.0-4.0 log units. With the vegetative cells, using the smallest support (25 µm) resulted in an average reduction of 4.0 log units. Conversely, lower antimicrobial activity was noted with the EOC-functionalized supports with a mean particle size of 200 µm. The sample flow through the supports with a large particle size (200 µm) was faster than filtering samples using small supports (25 µm), which cut the contact time between the target microorganism and the EOCs immobilized onto the supports' surface. These results agree with those obtained while evaluating the removal capability of the different supports against *B. subtilis* vegetative cells. The efficacy of the 200-µm supports could be increased by using a thicker filtering bed (Peña-Gómez et al., 2019b).

In the event of sporicidal activity of the EOC-functionalized supports, a great inhibitory activity was noted for both EOC-functionalized supports despite the particle size and inoculated media used (Figure 6).

The statistical data analysis (Table S3) confirmed that functionalization and particle size, and three interactions (functionalization-media, functionalization-particle size, functionalization-media-particle size), were the factors that significantly ($p<0.05$) influenced the supports' antimicrobial activity. Additionally, the factors that significantly ($p<0.05$) impacted the supports' sporicidal activity were functionalization, the media-particle size interaction and the interaction of all the factors.

Lastly, the fluorescent viability staining technique was also used to evaluate the viability of the cell/spores present in the recovery media. Figure S3 illustrates the viability fluorescence microscopy images of the *B. subtilis* cells and the spores present in the recovery media after

filtering samples with the bare and EOC-functionalized supports. It shows how the non-filtered samples and samples filtered through a bed of bare non-functionalized supports presented green-stained cells and spores, which confirm their viability, as well as the non-inhibitory activity of the silica microparticles. Conversely, after filtering the inoculated SW and NB/NB-Mn samples with either *B. subtilis* vegetative cells or spores through the thymol and vanillin EOC-functionalized supports, non-viable cells or spores (red-stained) or a few viable cells or spores (green-stained), were detected.

Therefore, given the results achieved in the present work, we can state that the antimicrobial and sporicidal activity of the EOC-coated filtering materials was proved by different viability assays. The antimicrobial and sporicidal effectiveness of the developed filtering materials could probably be explained by the combination of filters' physical adsorption given the electrostatic attractive forces between negatively charged microbial cell/spore surfaces and positively charged functionalized supports, and damage to microbial cell envelopes after coming into contact with the EOC immobilized on silica supports' surface during filtration (Peña-Gómez et al., 2019b; Pesce et al., 2014).

4 Conclusion

The developed silica supports coated with essential oil components (carvacrol, eugenol, thymol and vanillin) efficiently reduced *B. subtilis* vegetative cells and spores in filtered sterile distilled water and nutrient broth, which confirmed the excellent inhibitory properties of the filters against *B. subtilis* due to a combination of physical adsorption and inactivation by coming into contact with the immobilized antimicrobial compounds. The conclusions obtained in this manuscript are quite

relevant because they confirmed the irreversible effect on commonly resistant spores, positioning these materials as a potential sterilization methodology. This opens doors to new applications of the developed filtering materials in different beverages as an alternative or complementary preservation methodology to heat treatment. Besides, the use of developed filtering materials may be extended to other biologically important matrices and the designed immobilization techniques can be applied to create antimicrobial-coated surfaces with inhibitory properties by contact or antifouling activity, which can be used in many fields (food, biomedical, textile, etc.). Nevertheless, before these alternative filters are used in a real industrial environment, further studies should be carried out to confirm their safety (biocompatibility and toxicological studies), the influence on the properties of the treated drink, the shelf-life of filtering materials, the potential application of this technology in combination with other preservation techniques to enhance the removal capacity and performance under real operating conditions.

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Author Contributions

S. Ribes carried out the experiments, collected and analyzed data, interpreted the results, and drafted the manuscript. M. Ruiz-Rico designed the study, analyzed data, and drafted the

manuscript. J.M. Barat supervised the investigation, reviewed the manuscript, and acquired the funds.

Conflicts of Interest

The authors declare no conflict of interest.

References

Ahn, J., Lee, H.-Y., & Balasubramaniam, V. (2015). Inactivation of *Geobacillus stearothermophilus* spores in low-acid foods by pressure-assisted thermal processing. *Journal of the Science of Food and Agriculture,* 95(1), 174–178. https://doi.org/10.1002/jsfa.6700

Artíguez, M. L., & Martínez De Marañón, I. (2015). Inactivation of spores and vegetative cells of *Bacillus subtilis* and *Geobacillus stearothermophilus* by pulsed light. *Innovative Food Science and Emerging Technologies,* 28, 52–58. https://doi.org/10.1016/j.ifset.2015.01.001

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H. U., & Egli, T. (2007). Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Applied & Environmental Microbiology,* 73, 3283–3290. https://doi.org/10.1128/AEM.02750-06

Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2008). Inhibition of *Alicyclobacillus acidoterrestris* spores by natural compounds. *International Journal of Food Science & Technology*, 43(7), 1271–1275. https://doi.org/10.1111/j.1365-2621.2007.01604.x

Botequim, D., Maia, J., Lino, M. M. F., Lopes, L. M. F., Simões, P. N., Ilharco, L. M., & Ferreira, L. (2012). Nanoparticles and surfaces presenting antifungal, antibacterial and antiviral properties. *Langmuir,* 28(20), 7646–7656. https://doi.org/10.1021/la300948n

Cai, R., Zhang, M., Cui, L., Yuan, Y., Yang, Y., Wang, Z., & Yue, T. (2019). Antibacterial activity and mechanism of thymol against *Alicyclobacillus acidoterrestris* vegetative cells and spores. *LWT,* 105, 377–384. https://doi.org/10.1016/j.lwt.2019.01.066

Chemat, F., Zill-E-Huma, & Khan, M. K. (2011). Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry,* 18(4), 813–835. https://doi.org/10.1016/j.ultsonch.2010.11.023

Chen, F., Shi, Z., Neoh, K. G., & Kang, E. T. (2009). Antioxidant and antibacterial activities of eugenol and carvacrol-grafted chitosan nanoparticles. *Biotechnology & Bioengineering*, 104, 30–39. https://doi.org/10.1002/bit.22363

Cortezzo, D. E., Setlow, B., & Setlow, P. (2004). Analysis of the action of compounds that inhibit the germination of spores of *Bacillus* species. *Journal of Applied Microbiology*, 96(4), 725–741. https://doi.org/10.1111/j.1365-2672.2004.02196.x

Cronin, U. P., & Wilkinson, M. G. (2007). The use of flow cytometry to study the germination of *Bacillus cereus* endospores. *Cytometry Part A,* 71A(3), 143–153. https://doi.org/10.1002/cyto.a.20368

Devi, R., Alemayehu, E., Singh, V., Kumar, A., & Mengistie, E. (2008). Removal of fluoride, arsenic and coliform bacteria by modified homemade filter media from drinking water. *Bioresource Technology,* 99(7), 2269–2274. https://doi.org/10.1016/J.BIORTECH.2007.05.002

Espejo, G. G. A., Hernández-Herrero, M. M., Juan, B., & Trujillo, A. J. (2014). Inactivation of *Bacillus* spores inoculated in milk by Ultra High Pressure Homogenization. *Food Microbiology,* 44, 204–210. https://doi.org/10.1016/j.fm.2014.06.010

Fan, L., Hou, F., Muhammad, A. I., Ruiling, L. V., Watharkar, R. B., Guo, M., Ding, T., & Liu, D. (2019). Synergistic inactivation and mechanism of thermal and ultrasound treatments against *Bacillus subtilis* spores. *Food Research International,* 116, 1094–1102. https://doi.org/10.1016/j.foodres.2018.09.052

García-Ríos, E., Ruiz-Rico, M., Guillamón, J. M., Pérez-Esteve, É., & Barat, J. M. (2018). Improved antimicrobial activity of immobilized essential oil components against representative spoilage wine microorganisms. *Food Control* 94, 177–186. https://doi.org/10.1016/J.FOODCONT.2018.07.005

Hyldgaard, M., Mygind, T., & Meyer, R. L. (2012). Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. *Frontiers in Microbiology,* 3, 1–24. https://doi.org/10.3389/fmicb.2012.00012

Koutchma, T. (2009). Advances in ultraviolet light technology for non-thermal processing of liquid foods. *Food and Bioprocess Technology*, 2(2), 138–155. https://doi.org/10.1007/s11947-008-0178-3

Krishna, V., Pumprueg, S., Lee, S. H., Zhao, J., Sigmund, W., Koopman, B., & Moudgil, B. M. (2005). Photocatalytic disinfection with titanium dioxide coated multi-wall carbon nanotubes. *Process Safety and Environmental Protection,* 83(4B), 393–397. https://doi.org/10.1205/psep.04387

Lawrence, H. A., & Palombo, E. A. (2009). Activity of essential oils against *Bacillus subtilis* spores. *Journal of Microbiology and Biotechnology,* 19(12), 1590–1595. https://doi.org/10.4014/jmb.0904.04016

Milani, E. A., & Silva, F. V. M. (2017). Ultrasound assisted thermal pasteurization of beers with different alcohol levels: Inactivation of *Saccharomyces cerevisiae* ascospores. *Journal of Food Engineering*, 198, 45–53. https://doi.org/10.1016/j.jfoodeng.2016.11.015

Onnis-Hayden, A., Hsu, B. B., Klibanov, A. M., & Gu, A. Z. (2011). An antimicrobial polycationic sand filter for water disinfection. *Water Science and Technology,* 63(9), 1997–2003. https://doi.org/10.2166/wst.2011.440

Park, H. S., Yang, J., Choi, H. J., & Kim, K. H. (2017). Effective thermal inactivation of the spores of *Bacillus cereus* biofilms using microwave. *Journal of Microbiology and Biotechnology,* 27, 1209–1215. https://doi.org/10.4014/jmb.1702.02009

Peña-Gómez, N., Ruiz-Rico, M., Fernández-Segovia, I., & Barat, J. M. (2019a). Study of apple juice preservation by filtration through silica microparticles functionalized with essential oil components. *Food Control,* 106, 106749. https://doi.org/10.1016/j.foodcont.2019.106749

Peña-Gómez, N., Ruiz-Rico, M., Pérez-Esteve, É., Fernández-Segovia, I., & Barat, J. M. (2019b). Novel antimicrobial filtering materials based on carvacrol, eugenol, thymol and vanillin immobilized on silica microparticles for water treatment. *Innovative Food Science and Emerging Technologies*, 58, 102228. https://doi.org/10.1016/j.ifset.2019.102228

Peña-Gómez, N., Ruiz-Rico, M., Pérez-Esteve, É., Fernández-Segovia, I., & Barat, J. M. (2020). Microbial stabilization of craft beer by filtration through silica supports functionalized with essential oil components. *LWT,* 117, 108626. https://doi.org/10.1016/j.lwt.2019.108626

Pesce, G., Rusciano, G., Sasso, A., Isticato, R., Sirec, T., & Ricca, E. (2014). Surface charge and hydrodynamic coefficient measurements of *Bacillus subtilis* spore by optical tweezers. *Colloids and Surfaces B: Biointerfaces,* 116, 568–575. https://doi.org/10.1016/j.colsurfb.2014.01.039

Pinho, S. C., Nunes, O. C., Lobo-da-Cunha, A., & Almeida, M. F. (2015). Inactivation of *Geobacillus stearothermophilus* spores by alkaline hydrolysis applied to medical waste treatment. *Journal of Environmental Management*, 161, 51–56. https://doi.org/10.1016/j.jenvman.2015.06.045

Pinto, C. A., Santos, M. D., Fidalgo, L. G., Delgadillo, I., & Saraiva, J. A. (2018). Enhanced control of *Bacillus subtilis* endospores development by hyperbaric storage at variable/uncontrolled room temperature compared to refrigeration. *Food Microbiology,* 74, 125–131. https://doi.org/10.1016/j.fm.2018.03.010

Reineke, K., Ellinger, N., Berger, D., Baier, D., Mathys, A., Setlow, P., & Knorr, D. (2013). Structural analysis of high pressure treated *Bacillus subtilis* spores. *Innovative Food Science & Emerging Technologies,* 17, 43–53. https://doi.org/10.1016/j.ifset.2012.10.009

Reverter-Carrión, L., Sauceda-Gálvez, J. N., Codina-Torrella, I., Hernández-Herrero, M. M., Gervilla, R., & Roig-Sagués, A. X. (2018). Inactivation study of *Bacillus subtilis*, *Geobacillus*

stearothermophilus, *Alicyclobacillus acidoterrestris* and *Aspergillus niger* spores under Ultra-High Pressure Homogenization, UV-C light and their combination. *Innovative Food Science and Emerging Technologies,* 48, 258–264. https://doi.org/10.1016/j.ifset.2018.06.011

Ribes, S., Ruiz-Rico, M., Pérez-Esteve, É., Fuentes, A., & Barat, J. M. (2019). Enhancing the antimicrobial activity of eugenol, carvacrol and vanillin immobilized on silica supports against *Escherichia coli* or *Zygosaccharomyces rouxii* in fruit juices by their binary combinations. *LWT,* 113, 108326. https://doi.org/10.1016/j.lwt.2019.108326

Soni, A., Oey, I., Silcock, P., & Bremer, P. (2016). *Bacillus* spores in the food industry: A review on resistance and response to novel inactivation technologies. *Comprehensive Reviews in Food Science and Food Safety,* 15(6), 1139–1148. https://doi.org/10.1111/1541-4337.12231

Valero, M., & Giner, M. J. (2006). Effects of antimicrobial components of essential oils on growth of *Bacillus cereus* INRA L2104 in and the sensory qualities of carrot broth. *International Journal of Food Microbiology,* 106(1), 90–94. https://doi.org/10.1016/j.ijfoodmicro.2005.06.011

Voundi, S. O., Nyegue, M., Lazar, I., Raducanu, D., Ndoye, F. F., Stamate, M., & Etoa, F.-X. (2015). Effect of essential oils on germination and growth of some pathogenic and spoilage spore-forming bacteria. *Foodborne Pathogens and Disease,* 12(6), 551–559. https://doi.org/10.1089/fpd.2014.1892

Wang, H., Gu, L., Lin, Y., Lu, F., Meziani, M. J., Luo, P. G., Wang, W., Cao, L., & Sun, Y. P. (2006). Unique aggregation of anthrax (Bacillus anthracis) spores by sugar-coated single-walled carbon nanotubes. *Journal of the American Chemical Society,* 128(41), 13364–13365. https://doi.org/10.1021/ja065455o

Wang, L. H., Pyatkovskyy, T., Yousef, A., Zeng, X. A., & Sastry, S. K. (2020). Mechanism of *Bacillus subtilis* spore inactivation induced by moderate electric fields. *Innovative Food Science & Emerging Technologies,* 62, 102349. https://doi.org/10.1016/j.ifset.2020.102349

Wells-Bennik, M. H. J., Janssen, P. W. M., Klaus, V., Yang, C., Zwietering, M. H., & Den Besten, H. M. W. (2019). Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions. *International Journal of Food Microbiology,* 291, 161–172. https://doi.org/10.1016/j.ijfoodmicro.2018.11.005

Wuytack, E. Y., & Michiels, C. W. (2001). A study on the effects of high pressure and heat on *Bacillus subtilis* spores at low pH. *International Journal of Food Microbiology,* 64(3), 333–341. https://doi.org/10.1016/S0168-1605(00)00478-5

Xiong, B., Piechowicz, B., Wang, Z., Marinaro, R., Clement, E., Carlin, T., Uliana, A., Kumar, M., & Velegol, S. B. (2018). *Moringa oleifera* f-sand filters for sustainable water purification. *Environmental Science and Technology Letters,* 5(1), 38–42. https://doi.org/10.1021/acs.estlett.7b00490

Zhao, D., Barrientos, J. U., Wang, Q., Markland, S. M., Churey, J. J., Padilla-Zakour, O. I., Worobo, R. W., Kniel, K. E., & Moraru, C. I. (2015). Efficient reduction of pathogenic and spoilage microorganisms from apple cider by combining microfiltration with UV treatment. *Journal of Food Protection,* 78(4), 716–722. https://doi.org/10.4315/0362-028X.JFP-14-452

Tables

Table 1. Degree of functionalization of the EOC-functionalized supports according to organic matter content (α) and density of EOCs on the particles' surface.

EOC-functionalized support	α (g organic matter/g solid)	Coating density (molec/nm^2)
Car-25 µm	0.0676	248.4
Eug-25 µm	0.0740	273.7
Thy-25 µm	0.0697	252.6
Van-25 µm	0.1577	663.2
Car-200 µm	0.0829	4079.1
Eug-200 µm	0.1003	5081.9
Thy-200 µm	0.0832	4730.6
Van-200 µm	0.1753	8867.3

Figures

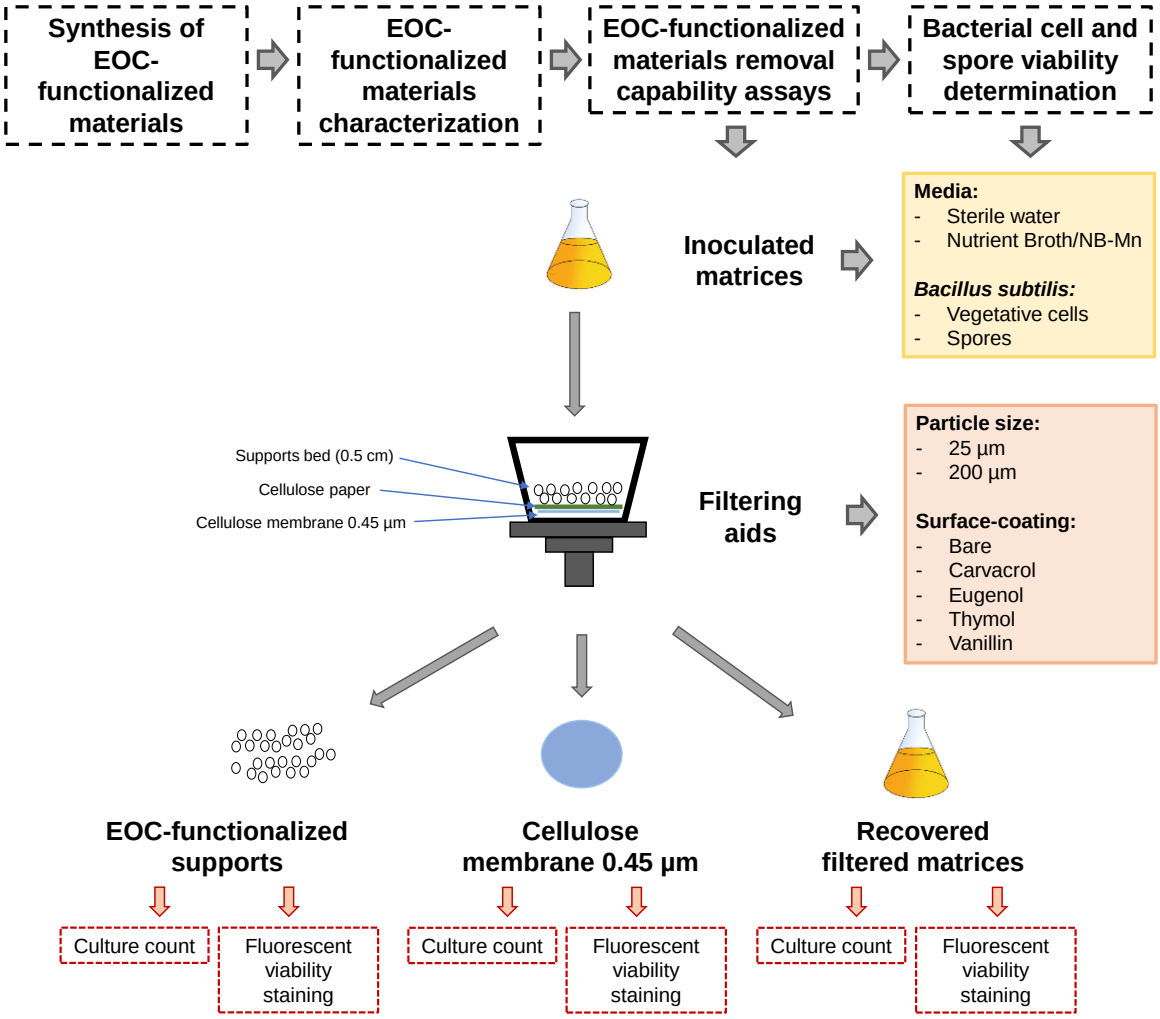
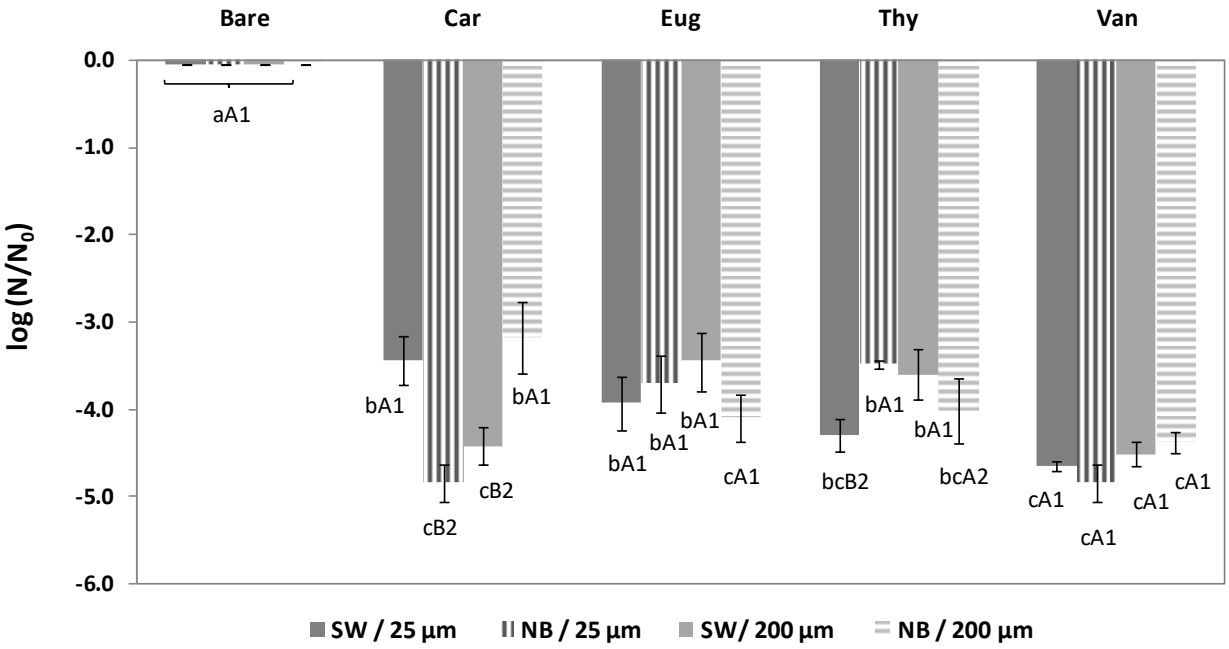


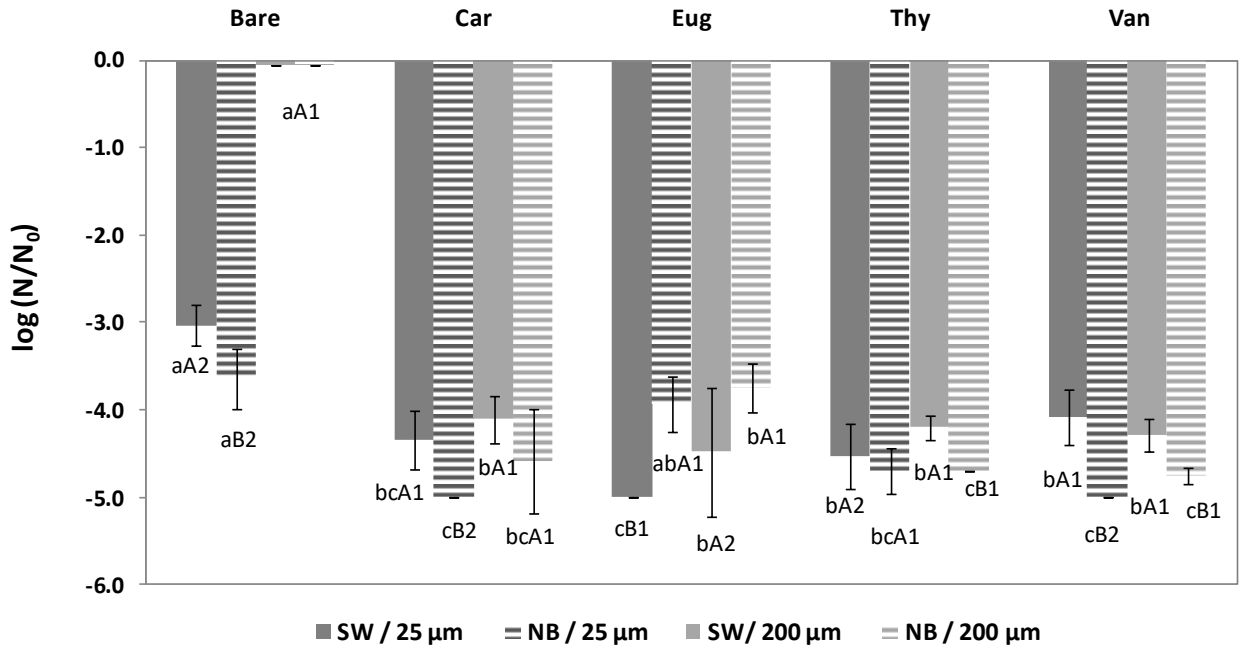
Figure 1. Schematic representation of the experimental procedure.



642

643 **Figure 2.** Removal capability (log (N/N_0)) of the bare and EOC-functionalized materials against *B.*
644 *subtilis* vegetative cells inoculated in sterile water (SW) or nutrient broth (NB) after a single
645 filtration through a bed (0.5 cm thick) of supports (25 µm and 200 µm). Mean values (n=3) ± SD.
646 Lowercase letters (a, b, c) indicate statistically significant differences among immobilized
647 compounds ($p<0.05$). Capital letters (A, B) denote statistically significant differences between
648 liquid matrices ($p<0.05$). Numbers (1, 2) indicate statistically significant differences between
649 particle sizes ($p<0.05$).

650



651

652 **Figure 3.** Removal capability (log (N/N_0)) of the bare and EOC-functionalized materials against *B.*
653 *subtilis* spores inoculated in sterile water (SW) or nutrient broth supplemented with $MnSO_4$ (NB-
654 Mn) after a single filtration through a bed (0.5 cm thick) of supports (25 µm and 200 µm). Mean
655 values (n=3) ± SD. Lowercase letters (a, b, c) indicate statistically significant differences among
656 immobilized compounds (*p<0.05*). Capital letters (A, B) denote statistically significant differences
657 between liquid matrices (*p<0.05*). Numbers (1, 2) indicate statistically significant differences
658 between particle sizes (*p<0.05*).

659

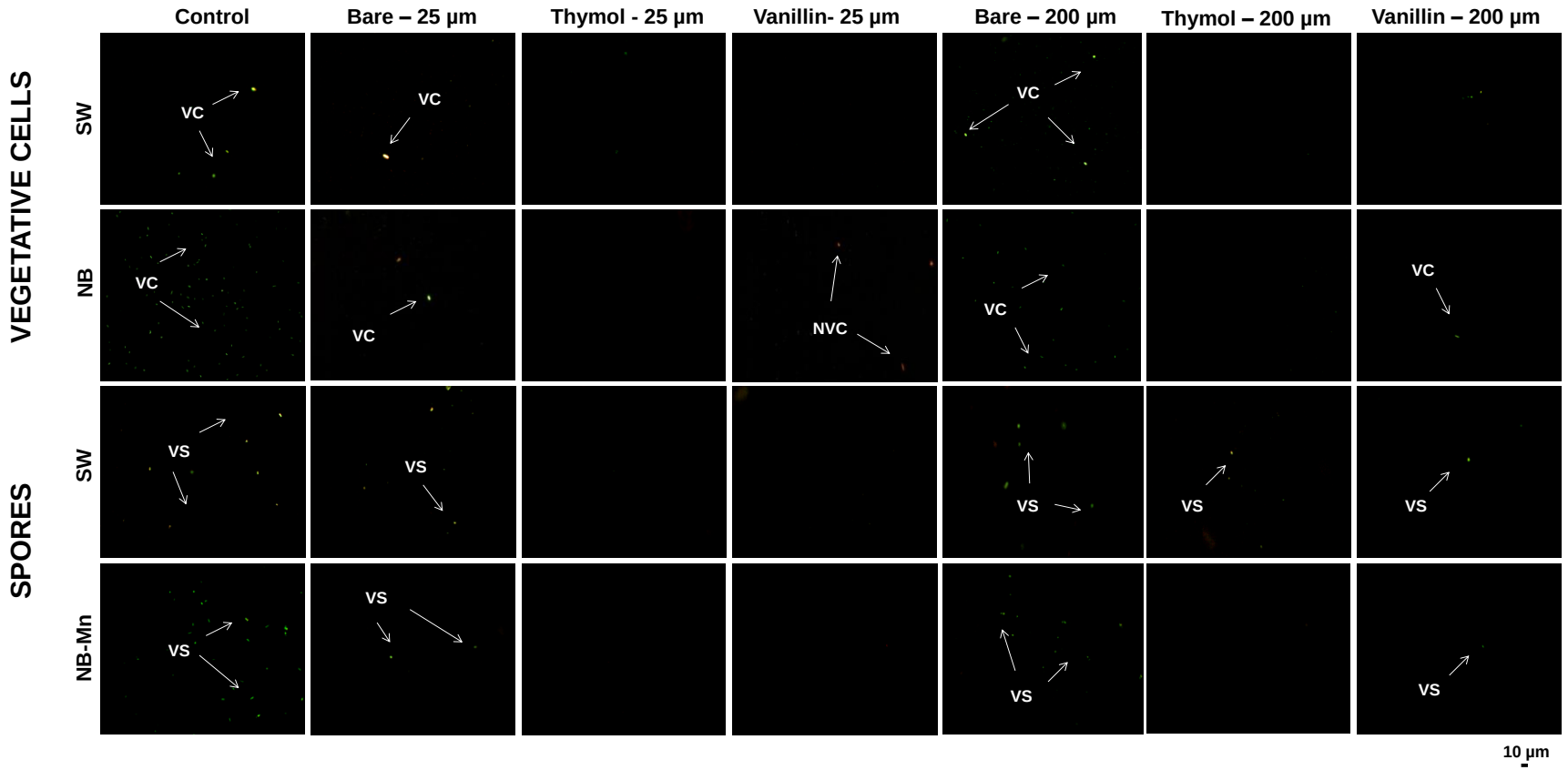


Figure 4. Fluorescence viability images of the *B. subtilis* vegetative cells and spores retained on cellulose membrane filters from the non-filtered (control) and filtered sterile water (SW) and nutrient broth (NB/NB-Mn) samples through the bed (0.5 cm thick) of the bare and EOC-functionalized supports (25 µm and 200 µm). VC: viable cells (green-stained cells); NVC: non-viable cells (red-stained cells); VS: viable non-germinated/germinated endospores (green-stained spores). Fluorescence images at 100x magnification.

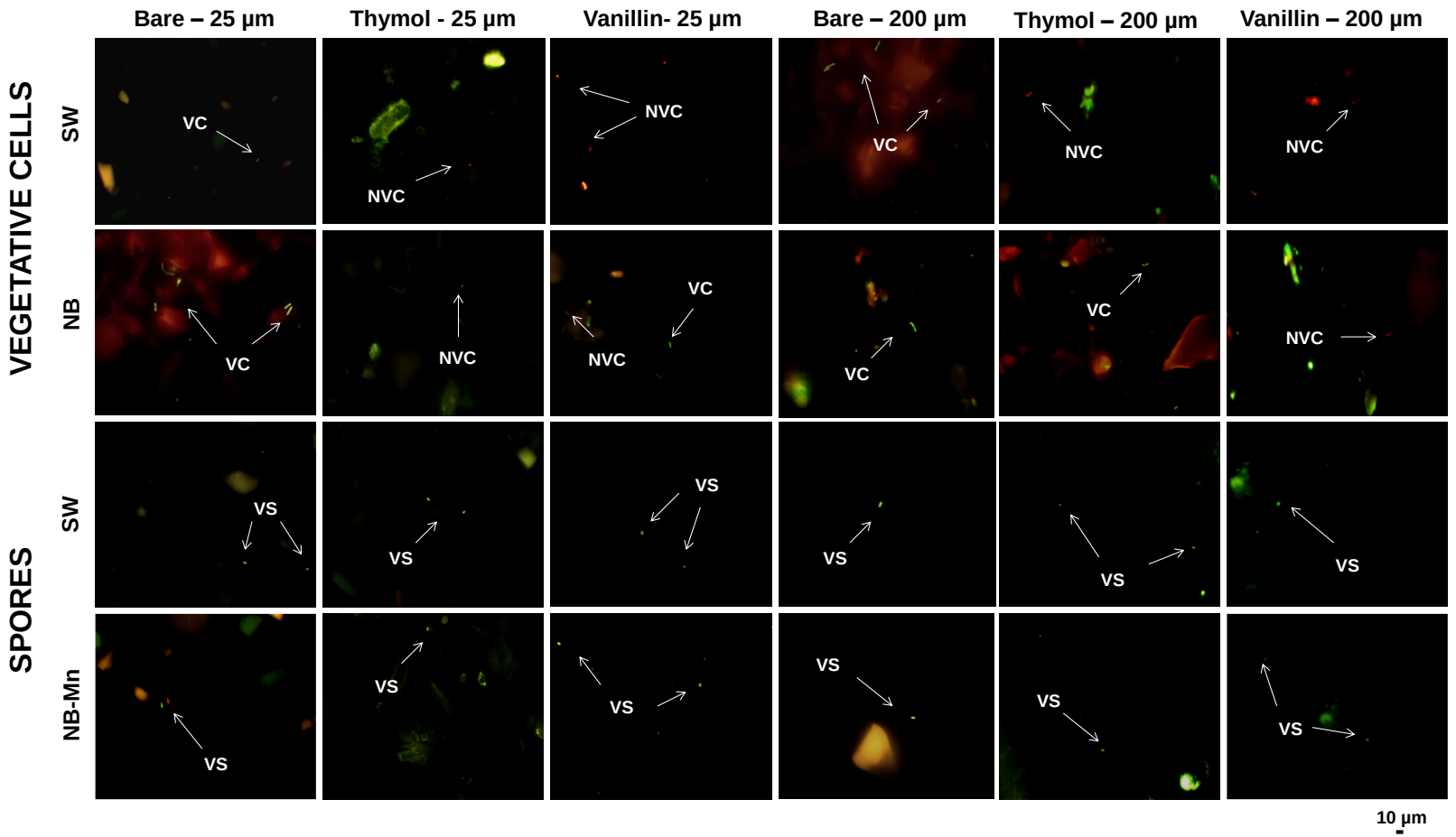


Figure 5. Fluorescence viability images of the *B. subtilis* vegetative cells and spores retained on the bed of the bare and EOC-functionalized supports after filtering the sterile water (SW) and nutrient broth (NB/NB-Mn) samples. VC: viable cells (green-stained cells); NVC: non-viable cells (red-stained cells); VS: viable non-germinated/germinated endospores (green-stained spores); NVS: non-viable non-germinated/germinated endospores (red-stained spores). Fluorescence images at 100x magnification.

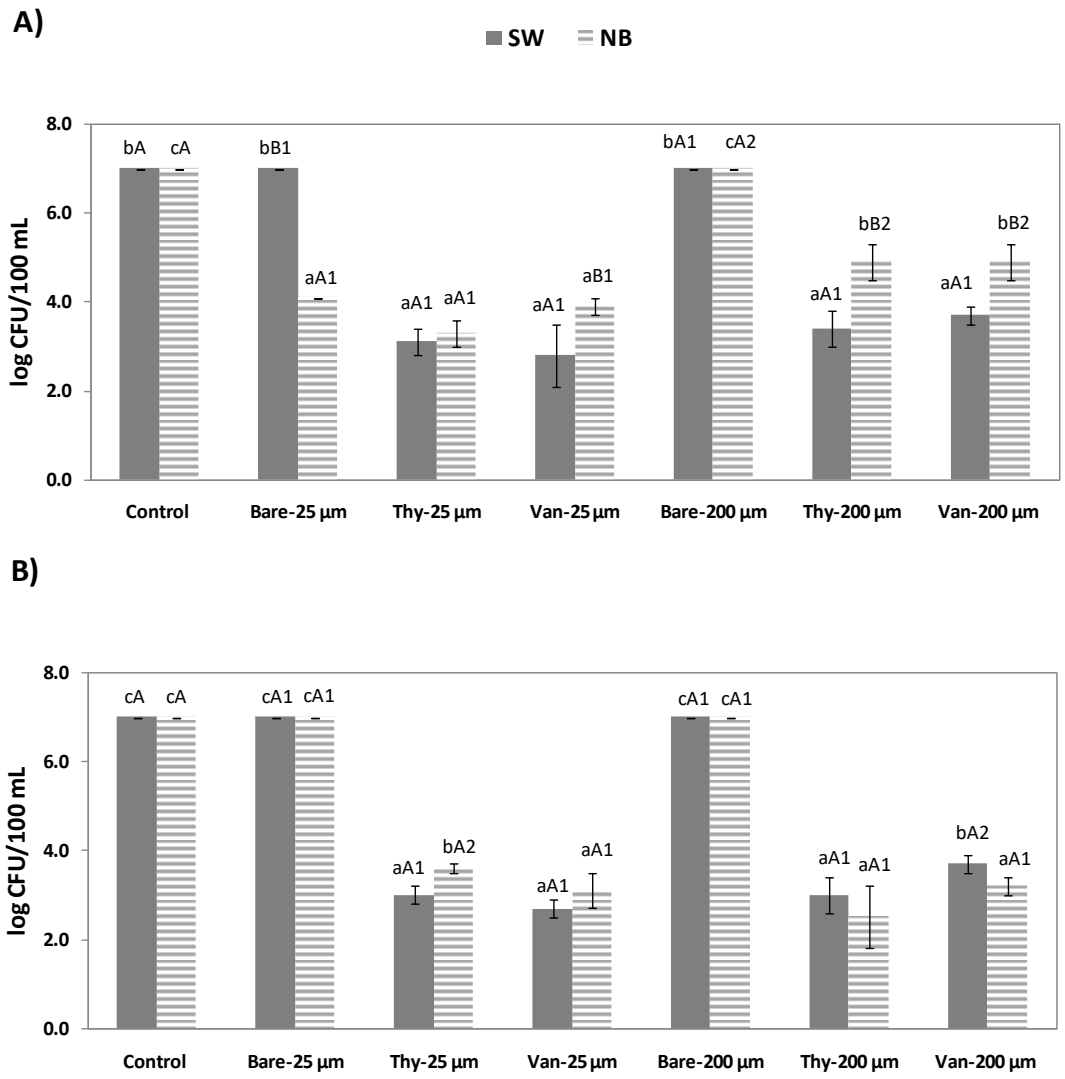


Figure 6. Plate counts of the *B. subtilis* vegetative cells (a) and spores (b) present in the recovery liquid medium after filtering the inoculated sterile water (SW) and nutrient broth (NB/NB-Mn) samples through the bed (0.5 cm thick) of the bare and EOC-functionalized supports (25 µm and 200 µm). Non-filtered water samples were labelled as controls. Lowercase letters (a, b, c) indicate statistically significant differences among immobilized compounds ($p<0.05$). Capital letters (A, B) denote statistically significant differences between liquid matrices ($p<0.05$). Numbers (1, 2) indicate statistically significant differences between particle sizes ($p<0.05$).