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In vitro* antimicrobial activity of immobilised essential oil components against *Helicobacter pylori

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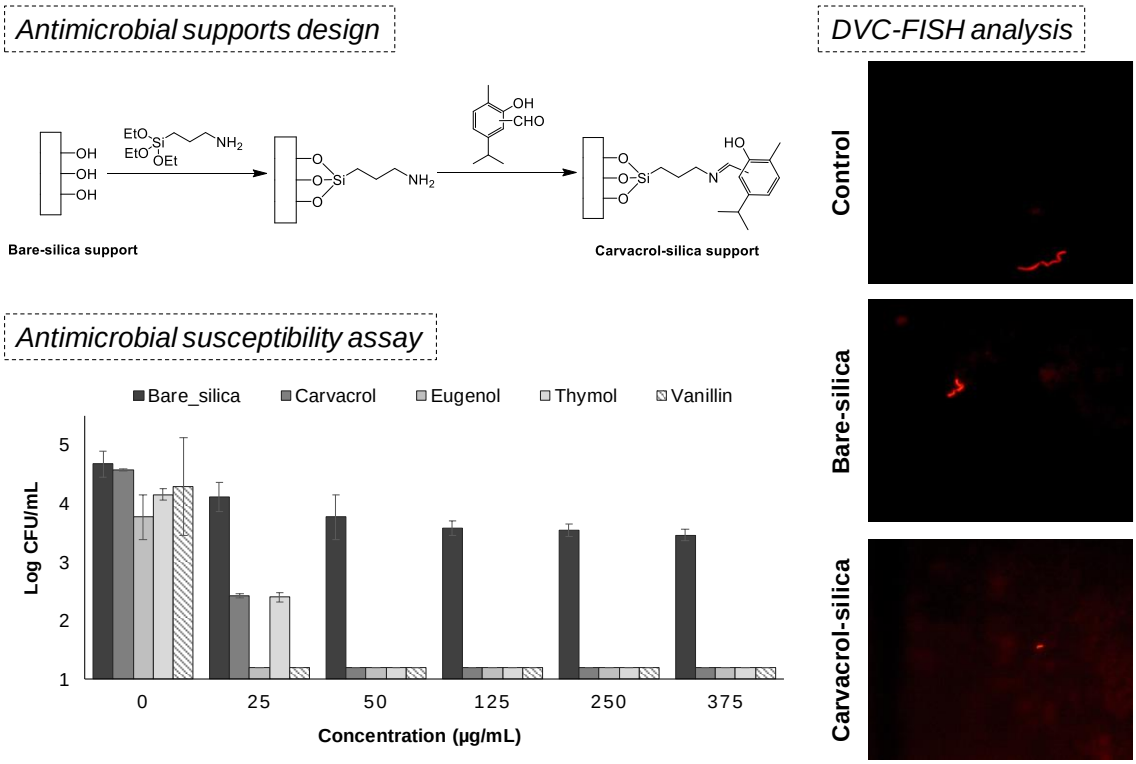

Graphical abstract

Abstract

Available disinfection methods and therapies against *Helicobacter pylori* have multiple disadvantages, such as increased prevalence of antibiotic-resistant strains, which requires the search for novel effective antimicrobial agents against *H. pylori*. Among them, naturally-occurring antimicrobial compounds, like essential oil components, have been reported as substances with anti-*H. pylori* potential. To avoid the disadvantages associated with using essential oil components in their free form, including volatility, low water solubility and intense sensory properties, their immobilisation in inert supports has recently been developed. This study sought to evaluate the inhibitory properties of essential oil components immobilised on silica microparticles against *H. pylori* and to elucidate the mechanism of action of the immobilised antimicrobials. After the preparation and characterisation of the antimicrobial supports, the susceptibility of *H. pylori* in the presence of the immobilised compounds was assessed by plate count, fluorescent viability staining and DVC-FISH analyses. The antimicrobial supports were found to inhibit *H. pylori* growth, and to induce morphological and metabolic alterations to the *H. pylori* membrane, with a minimum bactericidal concentration (MBC) value between 25-50 μg/ml according to the tested EOC. These findings indicate that immobilised essential oil components can be used as potential antimicrobial agents for *H. pylori* clearance and treatment.

Keywords: antimicrobial agent; *Helicobacter pylori*; immobilization; essential oil components; silica microparticles.

Introduction

Helicobacter pylori, a pathogenic Gram-negative microorganism, is considered the most extended infectious agent in humans that results in approximately 50% of the world's infected population (Wang et al. 2015). This bacterium substantially produces urease, which converts urea into ammonia and leads to low stomach acidity that allows its survival in the stomach and duodenum regions (Wolf et al. 2017). The presence of high-density *H. pylori* in gastric mucosa is associated with severe gastritis, an increased incidence of peptic and duodenal ulcers, and is a risk factor in the development of gastric mucosa-associated lymphoid tissue lymphoma and adenocarcinoma (Wang et al. 2015).

The routes of *H. pylori* transmission are still unclear, but both the oral-oral and faecal-oral routes seem to be involved (Nilsson et al. 2002). The faecal-oral route can include the intake of contaminated water or food that favours the environmental transmission of the infection (Eusebi et al. 2014). *H. pylori* can survive in the biofilms that form in water supply distribution systems by persisting with low levels of residual chlorine for a long time (Santiago et al. 2015). In fact *H. pylori* has been identified in chlorinated tap water and drinking water treatment plants (DWTP) (Santiago et al. 2015; Vesga et al. 2018).

Treatment of infection requires multidrug therapies. However, eradicating *H. pylori* is still a challenge given the increased prevalence of antibiotic-resistant *H. pylori* strains (Fuccio et al. 2008; Ardalan et al. 2017). Therefore, the search for novel effective antimicrobial agents against *H. pylori* is essential (Ali et al. 2005). Among them, different natural products and food components have been reported as substances with anti-*Helicobacter pylori* potential, such as polyphenols, lactoferrin, phenolic acids or essential oils (Takeuchi et al. 2014).

Essential oil components (EOCs) are secondary metabolites for plant defence with recognised antibacterial, antifungal, antiviral and antioxidant properties (Burt 2004; Nazzaro et al. 2013). Given their excellent antimicrobial function, EOCs possess high potential and have been proposed as natural additives for food preservation (Lv et al. 2011). However, the use of EOCs

as a food additive presents major limitations, such as high volatility (Burt 2004), poor water solubility (Burt et al. 2005), instability (Turek and Stintzing 2013), interaction with food components that results in higher concentrations, to achieve the antimicrobial effect in foods (Hyldgaard et al. 2012), as well as a strong organoleptic impact that can alter the taste of food and exceed the acceptable flavour threshold for consumers (Gutierrez et al. 2008).

Alternative technologies for the direct application of naturally-occurring antimicrobial compounds have been recently proposed, and have resulted in the design of new antimicrobial agents, such as encapsulation (Bernardos et al. 2015) or immobilisation in inert supports (Chen et al. 2009; Cappannella et al. 2016). The grafting of antimicrobial compounds onto substrates' surface will confer antimicrobial properties to widely applied materials by creating antimicrobial functionalised materials. Previous studies have demonstrated that the immobilisation process allows the antimicrobial power of the attached molecules to anchor, concentrate and improve (Li and Wang 2013; Ruiz-Rico et al. 2018), and can also prevent the limitations of free molecules. In line with this, we recently proposed the covalent immobilisation of EOCs on the surface of inert supports (Ruiz-Rico et al. 2017). The grafting of these bioactive compounds onto the surface of microparticles allowed us to improve the antimicrobial activity of free molecules against spoilage microorganisms (García-Ríos et al. 2018), and to mitigate their impact on food products (Ribes et al. 2017).

By taking into account the inhibitory properties of the immobilised EOCs against food-related microorganisms, it would be interesting to evaluate their effectiveness against an emerging pathogenic microorganism of great interest, namely *H. pylori*. Cellular viability can be addressed through different parameters like culturability ability, membrane integrity, respiratory activity, metabolic activity, etc. *H. pylori* is able to enter a viable, but non-culturable, state (VBNC) under stressed conditions. In this state, the pathogen loses its culturability, but remains active and, therefore, is potentially infective. To measure the viability of VBNC cells, culture-independent methods, such as direct staining using fluorescent markers SYTO/PI (the LIVE/DEAD BacLight

bacterial viability kit) to evaluate membrane integrity and the DVC-FISH technique to identify metabolic activity, have been successfully used (Adams et al. 2003; Moreno et al. 2007). The present study aimed to assess the antimicrobial properties of different essential oil components immobilised on silica microparticles against *H. pylori,* and to elucidate the mechanism of action of the immobilised bioactive molecules.

Materials and Methods

Reagents

Carvacrol (≥ 98% w/w), eugenol (99% w/w), thymol (≥ 98.5% w/w), (3-aminopropyl) triethoxysilane, trimethylamine, paraformaldehyde, chloroform, n-butanone and sodium borohydride were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile, methanol, diethyl ether, dichlorometane, NaCl, HCl, H_2SO_4, KOH and $MgSO_4$ were provided by Scharlab (Barcelona, Spain). Vanillin was obtained from Ventós (Barcelona, Spain). Amorphous silica microparticles (5 µm) were supplied by Silysiamont (Milano, Italy).

Antimicrobial supports preparation

The antimicrobial supports were synthesised following the methodology previously described by García-Ríos et al. (2018) with some modifications.

Aldehyde derivatives of carvacrol, eugenol and thymol were prepared to preserve the hydroxyl group of the essential oil components, which plays a key role in their antimicrobial activity (Burt 2004; Hyldgaard et al. 2012). Conversely, as vanillin possesses an aldehyde group in the chemical structure, the derivatisation procedure was not necessary. The aldehyde derivatives were synthesised by formylation reactions (Vilsmeier and Reimer-Tiemann) (Chen et al. 2009).

The aldehydes of carvacrol, eugenol and thymol and pure vanillin were reacted with (3-aminopropyl) triethoxysilane to obtain the corresponding alkoxysilane derivatives, which were immobilised onto the surface of silica microparticles. After anchoring the bioactive compounds

to the particles, the reduction of the imine bond to an amine bond was carried out with an excess of sodium borohydride. Then, the solids were washed with water (pH 4) and ethanol, and dried at room temperature for 12 h.

Antimicrobial supports characterisation

The non-functionalised and EOC-functionalised supports were characterised by standard techniques. Particle size distribution was established in phosphate buffer using a Malvern Mastersizer 2000 (Malvern Instruments, UK) by following the Mie Theory (refractive index of 1.45, absorption index of 0.1). Suspensions of particles were previously sonicated to avoid agglomeration of solids. The zeta potential (ζ-potential) was determined by a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The particle suspensions (1 mg/ml) in phosphate buffer were previously sonicated to prevent particle aggregation. The ζ-potential was calculated from the particle mobility values using the Smoluchowski model. Fourier transform infrared spectroscopy (FT-IR) was performed in a Bruker Infrared Spectroscopy Tensor 27 instrument (Massachusetts, US) between 4000 and 400 cm^{-1} in the transmittance mode. The degree of functionalisation was determined by thermogravimetric analyses (TGA) and elemental analyses. TGA determinations were made on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc., Schwarzenbach, Switzerland) using a heating programme that consisted of a heating ramp of 10°C/min from 25 to 800°C 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 the organic matter established by TGA should correspond to the sum of the content of C, H, O and N. 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.

Microbiological analysis

Culture conditions and bacterial strain

The reference strain, *H. pylori* 11637 NCTC, from the National Collection of Type Cultures (NCTC), was cultured in Blood Agar Base (Oxoid, Basingstoke, UK), supplemented with 10% defibrillated horse blood (Thermo Scientific, Oxoid, Basingstoke, UK) and incubated under microaerobic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen) in anaerobic jars (Campygen, Oxoid, Basingstoke, UK) at 37 °C for 48 h. Subsequently, cells were transferred to 10 ml PBS (130 mmol/l sodium chloride, 10 mmol/l sodium phosphate, pH 7.2) to achieve an inoculum density of 10^6 cells/ml. Cells were counted by fluorescent viability staining (LIVE/DEAD® BacLightTM, Life Technologies, Gaithersburg, USA), following the manufacturer´s protocol.

Antimicrobial susceptibility assay

The antimicrobial activity of EOCs was determined by the macrodilution method (CLSI 2018), within a range of concentrations of the immobilised EOCs of 0, 25, 50, 125, 250 and 375 µg/ml. The required amount of the antimicrobial supports was calculated according to the results of thermogravimetric and elemental analyses (*vide infra*). The antimicrobial supports were suspended in 15 ml PBS in Erlenmeyer flasks, and each one was inoculated with 100 µl of the above-described inoculum. Flasks were incubated by orbital stirring at 15 °C for 24 h under microaerobic conditions. The control positive samples (PBS inoculated with the microorganism without the antimicrobial supports) were used to quantify the microbial count in the absence of treatment. The support used for immobilisation was also tested as a negative control. All the treatments were performed in triplicate. After incubation, different aliquots were taken to evaluate *H. pylori* survival by culture methods, membrane integrity determination and metabolic activity identification by DVC-FISH.

Cell culture counts

After incubation, cultivable cell numbers were determined by plating 100 µl of different 10-fold serial dilutions of samples on the above-described selective media and incubating under microaerobic conditions at 37 °C for 48-72 h. Agar plates were examined for the presence of characteristic colonies (size 0.5–1.5 mm, rounded, convex and translucent). The cultivable viable cell numbers were expressed as $\log_{10}$ CFU/ml, with a limit of detection of 5 CFU/ml.

Bacterial viability determination by fluorescent viability staining

The antimicrobial activity of the immobilised EOCs was also evaluated by measuring culture viability in presence of the antimicrobial supports at the 0 h and 24 h incubation times. A two-colour fluorescent staining kit (LIVE/DEAD® BacLightTM; Life Technologies, Gaithersburg, MD, USA) was used to visualise the viable and dead microbial cells according to membrane cell integrity. One of the dyes, SYTO 9 (green-fluorescent nucleic acid stain), labels all the microbial cells with either intact or damaged membranes. In contrast, propidium iodide (red-fluorescent nucleic acid stain) penetrates only the bacteria with damaged membranes, which reduces SYTO 9 stain fluorescence when both dyes are present.

An aliquot of the antimicrobial-treated samples (250 µl) was mixed with 0.4 µl of SYTO 9/propidium iodide (ratio 1:1) and incubated for 10 min in the darkness at room temperature. Then 5 µl of stained cells were placed on a poly-L-lysine slide for immunofluorescence (Menzel-Gläser, Braunschweig, Germany), and the suspension was sealed with a coverslip. The preparation was incubated in the darkness for 5-10 min to allow microorganisms to adhere to slides. Slides were observed under an Olympus BX50 fluorescence microscope with a U-MWB filter (Olympus Co., Hamburg, Germany). Twenty fields were randomly counted.

DVC-FISH analysis

The DVC-FISH analysis was carried out to discriminate between viable and non-viable cells based on the combination of the direct viable count method (DVC) and fluorescent *in situ* hybridisation

(FISH). Aliquots of the control samples and the samples treated with 0.5 mg/ml of the silica supports (bare and functionalised) were prepared for the DVC-FISH analysis at the 0 h and 24 h incubation times in duplicate.

For DVC incubation, an aliquot of 1 ml of each sample was taken from the flasks and inoculated in 9 ml of Brucella broth, supplemented with 5% new-born calf serum (PAA Laboratories, Austria), 0.5 µg/ml novobiocin (Sigma-Aldrich, Missouri, USA) and 0.0025 mg/ml sodium pyruvate. The mixture was incubated under microaerobic conditions at 37 °C for 24 h. Then cells were recovered by centrifugation, washed with phosphate buffer and fixed with 4% paraformaldehyde (PFA) for 2 h at 4 °C. The fixed samples were centrifuged, washed with phosphate buffer, and resuspended in 1:1 phosphate buffer/ethanol (v/v).

After fixation, samples were hybridised with an *H. pylori*-specific probe (HPY-CTGGAGAGACTAAGCCCTCC-), designed by Moreno et al. (Moreno et al. 2003), and synthesised by EXIQON (Exiqon A/S Vedvaek, Denmark). For that, the fixed samples were pre-treated with 0.1% gelatine and dehydrated with successive solutions of 50%, 80%, and 100% ethanol. Samples were hybridised with 10 µl of hybridisation buffer (0.9 m/l NaCl, 0.01% SDS, 20 mm/l Tris–HCl and 40% formamide, pH 7.6), containing 50 ng of the probe, at 46 °C in the darkness for 2 h. Afterwards, slides were incubated in a washing solution (20 mm/l Tris–HCl, 0.01%, SDS, 5 mm/l EDTA) at 48 °C in the darkness for 15 min, washed and air-dried. The hybridised samples were prepared with vectashield® antifade mounting medium (Fisher Scientific, Madrid, Spain) between the cover and slide, and were visualised by fluorescence microscopy with Olympus BX 50 filters U- MWB, U- MWIG, and U- MWIB and an Olympus DP-10 camera. Viable cells (replicative ability) were identified as the cells that elongated by at least twice their original size.

Statistical analysis

The results obtained in the antimicrobial susceptibility assay of the immobilised bioactive compounds were analysed by a one-way analysis of variance (ANOVA) to evaluate the effect of

the bioactive agent concentrations on microbial count. The data obtained in the characterisation of the antimicrobial supports were also analysed by a one-way ANOVA to discriminate among samples. 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.

Results

Material characterisation

The EOC-functionalised solids were obtained by the covalent immobilisation of EOCs on the surface of the amorphous silica material. After the immobilisation procedure, particles were characterised by diverse instrumental techniques.

The Fourier transform infrared (FT-IR) spectra of all the solids within the 4000–400 cm^{-1} wavelength range are shown in Figure 1. Solids presented the typical IR absorption bands of siliceous materials, as seen in the non-functionalised support (curve a). These characteristic peaks consist in a band at 452 cm^{-1}, attributed to the vibration of the Si-O bonds, a band at 787 cm^{-1}, attributed to SiO_4 tetrahedrons, a band at 945 cm^{-1}, corresponding to the Si-OH groups, and a band at 1070 cm^{-1}, attributed to the bond stretching vibrations of Si-O-Si.

In contrast, the absorption bands of the EOC-functionalised materials indicate the grafting of the bioactive molecules on the particles' surface. The immobilisation of the phenolic compounds could be attributed to the bands due to presence of the hydroxyl group and the aromatic ring of EOCs. The shoulder band around 1220-1240 cm^{-1} corresponded to the bending vibrations of the phenolic hydroxyl group of the EOCs. Similarly, the characteristic stretching vibration absorption of the benzene ring corresponded to the increase in the band at 780 cm^{-1} (Peng et al. 2010). In addition, the broad band at 3000–3700 cm^{-1} was ascribed to the O-H bonding vibration of adsorbed water and SiO-H groups (Villalonga et al. 2013), the stretching vibration of O–H bonds

and the aromatic C–H of EOCs (Altiok et al. 2010) and the bending N–H vibrations of the amine groups of the trialkoxysilane derivatives.

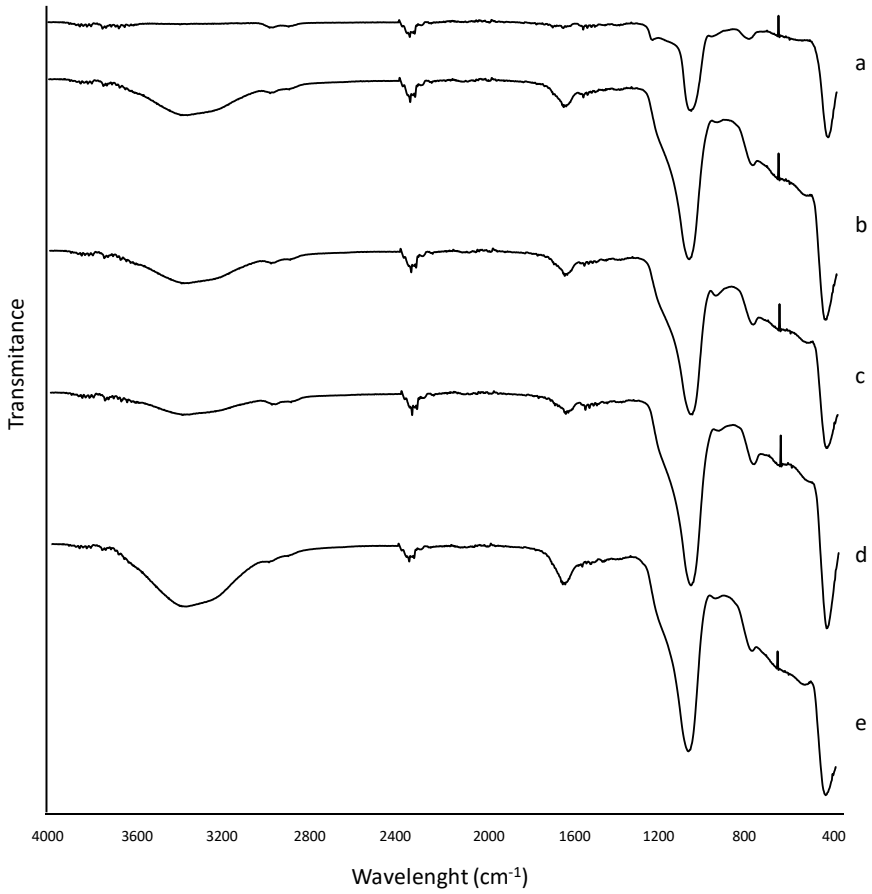

Figure 1. FT-IR spectroscopy of the non-functionalised amorphous silica microparticles (a), and the silica microparticles functionalised with carvacrol (b), eugenol (c), thymol (d) or vanillin (e).

Table 1 shows the degree of functionalization, particle size distribution ($d_{0.5}$), and ζ-potential of the bare and functionalized silica microparticles. The degree of functionalisation was quantitatively determined by thermogravimetric and elemental analyses to estimate the amount of the immobilised bioactive compounds onto the particles' surface. As we can see in the table, small amounts of carvacrol and thymol were attached, while the grafting of eugenol, and mainly that of vanillin, was more efficient. These values were used to calculate the amount of particles needed to study the equivalent concentrations of the immobilised EOCs.

Besides confirming the grafting of the antimicrobial molecules, particle size and the ζ-potential were determined. The bare and functionalised amorphous silica particles had a similar particle size to that reported in the technical data sheet of the material (ca. 5 μm). Non-significant differences were found between the bare and functionalised particles (p-value > 0.5), which supports the notion that the functionalisation process did not affect the particle size of the microparticles. The bare material obtained a negative ζ-potential value due to the presence of the silanol groups on the particle's surface. The ζ-potential of the EOC-functionalised particles changed to positive values (significant differences, p-value < 0.001), which confirmed the attachment of the bioactive compounds-alkoxysilane derivatives to the silica support surface (Ribes et al. 2017). With the change in the ζ-potential, the positively charged EOC-functionalised supports could show electrostatic attraction with the negatively charged bacterial surface, which would favour the supports' inhibitory capability (Ruiz-Rico et al. 2018).

Table 1. Content (α) in milligrams of EOCs per gram of SiO_2, particle size distribution ($d_{0.5}$) and the ζ-potential of the bare and EOC-functionalised amorphous silica particles (means and standard deviations, n=3).

Solid	**α (mg EOC/g SiO_2)**	**$d_{0.5}$ (μm)**	**ζ-potential (mV)**
Non-functionalised	-	5.8±0.3	-33.2±1.2[a]
Carvacrol	10.5	6.2±0.7	37.3±0.6[d]
Eugenol	16.0	5.9±0.4	30.3±2.6[c]
Thymol	9.2	6.4±2.1	22.9±4.1[b]
Vanillin	35.7	6.2±1.1	29.3±1.0[c]

Different letters in the same column indicate heterogeneous group membership (p<0.001)

Effect of antimicrobial supports on *H. pylori*

The *H. pylori* counts after treatment with carvacrol, eugenol, thymol and vanillin immobilised on the amorphous silica microparticles are shown in Figure 2. Equivalent amounts of the bare particles were included in the experiment to assess the influence of the support on the microorganism's viability. According to the culturable cell count results, the incubation of *H. pylori* with the non-functionalised support had a slight inhibitory effect. This behaviour agrees with previously reported results (Ruiz-Rico et al. 2017; García-Ríos et al. 2018). In contrast, a different reduction or inhibition of the microorganism was observed according to the immobilised bioactive compound and their concentration after a 24-hour incubation with the EOC-functionalised particles. *H. pylori* counts significantly lowered ($p<0.05$) as the concentration of the immobilised antimicrobials increased. The use of 25 μg/ml of immobilised carvacrol and thymol attained a *H. pylori* reduction of 2-log cycles, while microbial culturability was completely inhibited when the same concentrations of immobilised eugenol and vanillin were used. Higher concentrations of all the supports showed non-detectable levels of the cultivable microorganism 24 h after treatment began.

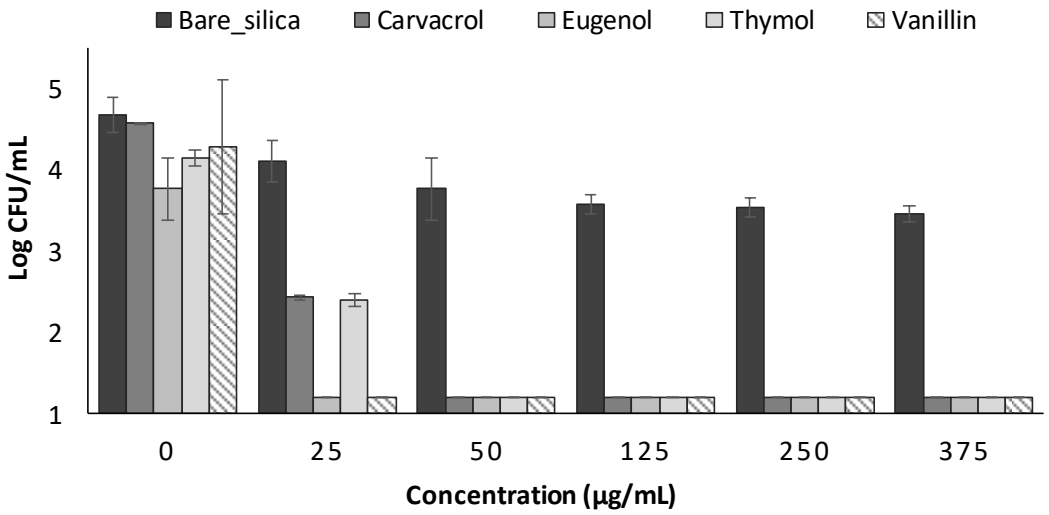

Figure 2. Culture (log CFU/ml) of *H. pylori* after the incubation with bare silica microparticles and immobilised carvacrol, eugenol, thymol and vanillin according to the EOC concentration (means and standard deviations, n=3).

Given the delicate and fastidious nature of *H. pylori*, the *in vitro* cultivation might not correctly define the inhibitory potential of an antimicrobial agent. Therefore, the antimicrobial activity of the immobilised EOCs was also evaluated by measuring cell viability with a fluorescent staining kit. The two-colour fluorescent LIVE/DEAD® BacLightTM assay visualised viable and dead *H. pylori* cells in the absence and presence of the bare and EOC-functionalised silica microparticles after 0 h and 24 h of incubation. The enumeration of viable cells using fluorescence dyes gave counts in the same order as the plate count results (data not shown). In general, the colour of the *H. pylori* cells remained green at the 0 h incubation time, which meant that cells were viable and membranes remained intact. In contrast, after 24 h of incubation with the EOC-functionalised particles, the absence of cells or the presence of red-coloured cells indicated a damaged cell envelope, and eventual bacterial death (Ruiz-Rico et al. 2018).

Lastly, the *H. pylori* viability was established by means of its metabolic activity following the DVC-FISH method. Figure 3 shows the images of the *H. pylori* cells in the samples either untreated or treated with the bare and EOC-functionalised silica supports after 0 h and 24 h of incubation. When DVC-FISH was applied, elongated or fattened spiral bacillus cells were observed, which are considered to be viable cells (Santiago et al. 2015), in the control sample and the sample incubated with the bare silica microparticles. However, the incubation of the samples in presence of the immobilised EOCs irreversibly affected cells' metabolic activity because, while the bacillary elongated cells were observed at 0 h, almost no cells or no elongated ones were detected after 24 h of incubation. In fact only coccoid forms (eugenol, vanillin) or short bacilly (carvacrol) were visualised.

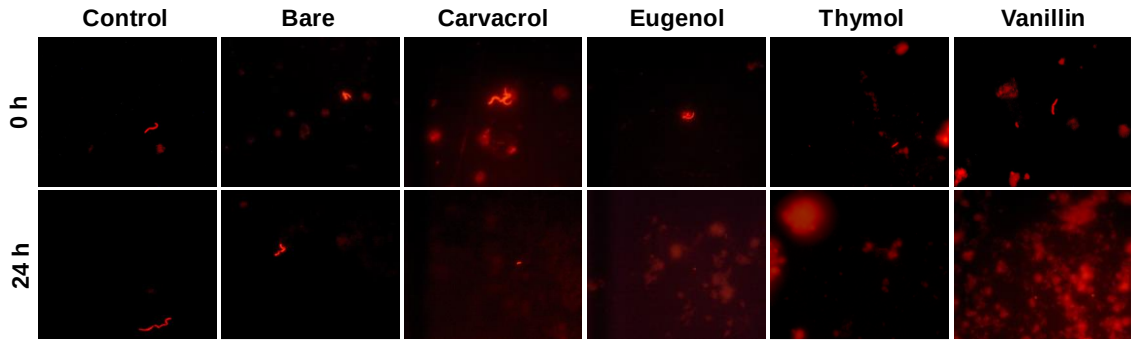

Figure 3. The DVC-FISH images of viable *H. pylori* in the control samples and the samples treated with the bare and EOC-functionalised silica supports at 0 and 24 h of incubation.

Discussion

Essential oil components present remarkable antimicrobial activity, are considered generally recognised as safe (GRAS) substances by the United States Food and Drug Administration (FDA) and have been accepted by the European Commission for their intended use as flavourings in food products (Hyldgaard et al. 2012).

Their immobilisation in biocompatible inert supports, such as silica microparticles, offers the advantage of avoiding strong sensory properties, poor solubility and instability of EOCs, and of preserving, or even improving, their antimicrobial activity against food spoilage microorganisms (García-Ríos et al. 2018). The grafting process was based on the covalent attachment of EOCs to the surfaces of the material substrate that bore surface hydroxyls. Hydroxyl moieties can be reacted with trialkoxysilane derivatives, which can contain several reactive organic functional groups that allow the direct linking of EOCs through the formation of covalent bonds (Hoffmann et al. 2006). The aminosilane (3-aminopropyl) triethoxysilane was used in this work as grafting agent because its possesses catalytic activity upon the formation of siloxane bonds with silanols on the surface of the substrate, and it also has attachment-free sites that can react with bioactive organic molecules (Kahraman et al. 2007).

The immobilisation of EOCs on silica supports allowed us to design novel antimicrobial agents. The incubation of *H. pylori* in the presence of the bare and EOC-functionalised supports resulted

in cellular viability loss and, thus, the microbial inhibition of the microorganism according to the plate count method.

In addition to this method, we used other techniques to determine the bacterial viability of *H. pylori* due to the potential acquisition of a physiological state in which they are viable, but cannot form colony-forming units (VBNC), so they would go undetected. These procedures included the assessment of membrane cell integrity by the LIVE/DEAD BacLight kit® and the evaluation of metabolic activity by the DVC-FISH technique. With these techniques, we also attempted to elucidate the mechanism of action of the antimicrobial supports against *H. pylori*.

Fluorescent viability staining with LIVE/DEAD® BacLightTM demonstrated the effect of the EOC-functionalised supports on the cell membrane due to the presence of high local concentrations of the naturally-occurring antimicrobial molecules on particles, which could easily disrupt the cell membrane and induce cell death (Li and Wang 2013; Ruiz-Rico et al. 2018). This experiment allowed the inhibitory properties of the immobilised EOCs to be ratified because non-significant differences were observed between the culturable cell counts and the viable counts by assuming that 'viable but non-culturable' (VBNC) cells were not present in the samples after the treatment with the antimicrobial supports.

Along with the cell envelope integrity evaluation, the influence of the antimicrobial agents on the metabolic activity of *H. pylori* by DVC–FISH proved the permanent damage of bacterial cells. The DVC method is based on incubating samples in the presence of nutrients and a single gyrase inhibitor. The antibiotic inhibits DNA synthesis and prevents cell division, but cells can metabolise nutrients and become elongated and/or fattened after incubation. FISH allows specific detection, given the hybridisation of the fluorescent DNA probe, along with the differentiation of the sizes of the viable and non-viable cells after elongation (Piqueres et al. 2006). Our results displayed elongated cells only in the untreated sample and the sample incubated with the bare silica microparticles given the active or reactivable cellular machinery that allowed viable cells to elongate (Santiago et al. 2015). In contrast, no elongated cells were

observed in the presence of the EOC-functionalised supports. This was also evidenced by the transformation of the shape of the *H. pylori* cells. When *H. pylori* is subjected to environmental stress, such as aerobiosis, temperature changes, extended incubation and antimicrobial treatment, spiral bacillus can be transformed into coccoid, which can be stated in the samples treated with eugenol and the vanillin-functionalised support. Such cells might be responsible for transmission in the environment and antibiotic treatment failures (Nilsson et al. 2002; Moreno et al. 2007). Nevertheless, the absence of elongated cells after DVC–FISH incubation evidenced the irreversible damage of bacterial cells after the treatment with the EOC-functionalised supports.

The immobilised EOCs displayed high antimicrobial activity against the pathogenic Gram-negative microorganism *H. pylori*, with a minimum bactericidal concentration (MBC) value lying between 25-50 μg/ml, according to the tested EOCs. Previous studies have reported the antimicrobial properties of these bioactive compounds in their free form against *H. pylori*. Carvacrol showed *in vitro* antimicrobial activity against different strains of the target microorganism with an MBC value of 40 μg/ml (Bergonzelli et al. 2003). Similarly, eugenol and thymol displayed an antimicrobial effect that resulted in an MIC value of 2 μg/ml for eugenol (Ali et al. 2005) and MBC values of 100 μg/ml for both compounds (Bergonzelli et al. 2003). The use of an essential oil, like cinnamon oil, in the treatment of different *H. pylori* strains also exhibited inhibitory properties, but at higher concentrations (MIC 140-72,000 μg/ml) (Ardalan et al. 2017). Other naturally-occurring antimicrobial compounds of plant origin have shown an inhibitory effect against *H. pylori*. Gallic acid has obtained an MIC value over 1,000 μg/ml, whereas an ester of gallic acid, such as octyl gallate, a widely used antioxidant food additive, has shown greater antimicrobial activity with an MIC value of 125 μg/ml (Wolf et al. 2017). These results are generally higher than the antimicrobial properties obtained herein. Therefore, the improved antimicrobial properties of EOCs after their grafting to the surface of silica microparticles is implied in the present work. According to the results from the different

parameters of viability evaluated, such as culturability, membrane integrity and metabolic activity, the mechanism of action of these compounds seems to affect different cell physiological properties.

The present results can be taken as a starting point that might be significant to prevent and treat *H. pylori* infections given the difficulties in designing prevention measures due to limited knowledge on transmission pathways (Nilsson et al. 2002), the importance of the risk of developing associated pathologies (Ali et al. 2005), and the limitations of current treatments (Fuccio et al. 2008).

Conclusions

In this work the antimicrobial properties of naturally-occurring antimicrobial compounds immobilised on silica microparticles against *H. pylori* have been demonstrated in culturability, viability and metabolic activity terms. Indeed the results indicate that the immobilised antimicrobials irreversibly affected *H. pylori* viability at different structural and physiological cell levels. The developed antimicrobial supports are based on the combination of natural molecules and biocompatible supports, which allows safe antimicrobial agents to be designed that can be used to treat water and food products, and to prevent antibiotic-resistant strains from spreading.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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