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Non-thermal inactivation of Alicyclobacillus acidoterrestris and guaiacol production in orange juice by using silica microparticles functionalised with essential oil components

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ABSTRACT

Alicyclobacillus acidoterrestris is an acidophilic, spore-forming bacterium that is particularly notable for its ability to spoil fruit juice and other acidic beverages. It produces guaiacol, a compound that confers these products a smoky, medicinal or phenolic off-flavour, which results in consumer rejection and significant economic losses for the beverage industry. This microorganism is also known for its high thermal resistance, which poses challenges in traditional thermal preservation methods. This work aimed to assess the effect of silica microparticles functionalised with essential oil components on controlling the growth of vegetative cells of different *A. acidoterrestris* strains and subsequent guaiacol production capacity in orange juice. Immobilised essential oil components were applied following two different approaches: contact with juice in uniform dispersion; filtering juice through a layer of functionalised microparticles. Firstly, the potential of the different pure essential oil components was evaluated by *in vitro* experiments in culture medium by determining microbial counts and guaiacol production by HPLC. These tests revealed that eugenol and thymol were the most effective antimicrobials, while vanillin and geraniol displayed moderate or minimal effectiveness, respectively. The orange juices inoculated with *A. acidoterrestris* were treated by contact or filtration with both the free and immobilised forms of eugenol and thymol. In the first approach, the juice incubation with MIC × 0.5 of free or immobilised eugenol and thymol for 24 h lowered *A. acidoterrestris* counts and inhibited guaiacol production. In the second approach, the inoculated juices were also filtered through eugenol and thymol particles, and this fast process was able to inhibit guaiacol production. These findings highlight the potential of harnessing the antimicrobial properties of immobilised essential oil components to mitigate *A. acidoterrestris* contamination risks during juice production. By these approaches, juice producers can meet consumer demands for safe, premium-quality products, while ensuring extended shelf life and minimising flavour defects and, thus, food waste.

1. Introduction

The food industry is currently facing several challenges, especially in quality preservation, microbiological safety and food waste reduction areas (Zhang et al., 2023). This is due mostly to the perishable nature of food products, more consumers preferring natural and minimally processed options, and growing environmental awareness about heat treatments, which usually implies the release of greenhouse gas emissions and, consequently, contributes to global warming (Panigrahi et al., 2021). Despite considerable efforts carried out in last years, the Food and Agriculture Organization (FAO) reports staggering figures, with estimates showing that around 1.3 billion tons of agricultural products are wasted annually (FAO The State of Food and Agriculture, 2019). In addition, the study of Mudaliar et al. (2023) highlights a significant problem for postharvest industries by estimating that 25% of harvests (fruit, vegetables, and their by-products) is lost due to microbial contamination. This issue is particularly relevant in juice production industries, where spoilage microorganisms can rapidly degrade product

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quality, which leads to substantial economic losses (Bigi et al., 2023).

Of the microorganisms that compromise juice products' quality, *Alicyclobacillus acidoterrestris* stands out and is the cause of significant concern (Kaur et al., 2024; Wahia et al., 2022). The first documented case of spoilage by this microorganism dates back to 1982 (Cerny et al., 1984). Despite its long-standing presence, detecting *A. acidoterrestris* in food is challenging because it neither alters contaminated products' colour or texture, nor produces observable gases that can cause package swelling (Sinigaglia et al., 2003). However, the *A. acidoterrestris* bacterium is able to metabolise certain compounds present in juices, which results in undesirable flavours that render the product unsuitable for consumption. Of all the metabolites produced by *A. acidoterrestris*, guaiacol is widely recognised as a key compound involved in quality loss by conferring contaminated products a smoky or medicinal off-flavour. According to Cai et al. (2015), guaiacol synthesis by *A. acidoterrestris* uses vanillin, a compound that is naturally present in orange juice (Goodner et al., 2000), as a substrate. As Fig. 1 illustrates, synthesis is carried out by specific enzymatic reactions in two steps. Initially, vanillin dehydrogenase converts vanillin into vanillic acid. Subsequent decarboxylation by vanillin acid decarboxylase produces guaiacol. By taking all these facts into account, controlling *A. acidoterrestris* growth and minimising guaiacol production, which are recognised key indicators of *A. acidoterrestris* activity in orange juice, are critical for preserving juice products' sensory properties and consumer attractiveness, and fall in line with the priority of maintaining product quality (Pérez-Cacho et al., 2011).

Many approaches have been described to control *Alicyclobacillus* spp. contamination, but lots of them are based on heat treatments and are ineffective because this bacterium is highly thermoresistant (Wahia et al., 2022). Alternative methods, such as high hydrostatic pressure (Georget et al., 2015), ultrasound or pulsed electric field treatments (Uemura et al., 2009), or photosensitizers and light sources (Ding et al., 2024; do Prado-Silva et al., 2024) have been reported to be effective, but are often hindered by associated costs (Putnik et al., 2018).

To overcome the above limitations and in line with the current demand for food treated with natural products (Novais et al., 2022), plenty of studies have paid attention to the use of purified plant extracts such as essential oil components (EOCs) (Wang et al., 2022). These compounds possess substantial broad-spectrum antimicrobial activity. Their use as preservatives is increasing. This is especially true because many of these components are recognised as being Generally Recognised as Safe (GRAS) by the U.S. Food and Drug Administration (Gómez-Llorente et al., 2023). Despite their effectiveness, the direct use of EOCs in food applications can have drawbacks, such as flavour modification, strong smell or low solubility in aqueous media, which justify the need for innovative solutions. To address these issues, the covalent immobilisation of EOCs onto inorganic supports as silica particles has been proposed in *in vivo* treatments against different microorganisms, including bacteria, fungi and viruses (Gómez-Llorente et al., 2024b; Peña-Gómez et al., 2019a; Peña-Gómez et al., 2019b; Ribes et al., 2017; Ruiz-Rico et al., 2021). This technique not only preserves the antimicrobial activity of EOCs, but also prevents them from leaching into the food matrix, which allows them to be reused.

The objective of this study was to assess the effect of EOCs immobilised on silica particles to control the growth of vegetative cells of different *A. acidoterrestris* strains and the subsequent guaiacol production capacity in orange juice by two different approaches: direct contact with juice in uniform dispersion and filtering juice through a layer of functionalised particles.

2. Material and methods

2.1. Materials

Eugenol (Eu, 99% w/w), geraniol (Ger, ≥98% w/w), thymol (Thy, ≥98.5% w/w), vanillin (Va, >99% w/w), (3-aminopropyl) triethoxysilane (APTES), formic acid and silica particles were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, methanol, acetonitrile, formaldehyde, peptone water, BAT *Alicyclobacillus* agar and BAT *Alicyclobacillus* broth were obtained from Scharlab (Barcelona, Spain). Sterilised orange juice made from concentrate (Consum, Valencia, Spain) was bought from a local supermarket. The five *A. acidoterrestris* strains used in this study were sourced from a private collection that had been previously isolated from commercial samples of orange juice (st1, st2, st3 and st4) and pear juice (st5). These strains have been isolated and characterised in an earlier study (Sánchez Sánchez, 2017).

2.2. In vitro susceptibility of A. acidoterrestris strains to different EOCs

In order to determine the antimicrobial susceptibility of each strain under study to the four proposed antimicrobial types, the microdilution technique was utilised to quantify the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of Eu, Ger, Thy and Va against the five *A. acidoterrestris* strains (Chandrasekaran & Venkatesalu, 2004). The MIC and MBC values were characterised as the minimum concentration at which bacterial growth was no longer evident and at which no microorganism growth was observed by plate counts, respectively (Owuama, 2017).

Before the study began, strains were thawed and reconstituted from cryovials according to the procedure reported by Tedeschi and De Paoli (2011). Bacterial cultures were prepared by two consecutive transfers in BAT broth at 46 °C for 48 h and seeded onto BAT agar. To obtain the bacterial inoculum, the cells from a colony were placed inside a test tube with 10 mL of BAT broth and were incubated at 46 °C for 48 h. The inoculum's cell concentration was determined by measuring the optical density at 620 nm using a Helios Zeta UV–VIS instrument (Thermo Scientific, Hampton, New Hampshire, USA).

For the MIC assay, the aliquots of the Eu, Ger, Thy and Va stocks were prepared by diluting DMSO stock solutions (400 mg/mL) in BAT growth, which yielded concentrations of 10, 5, 3.75, 2.5, 1 and 0.75 mg/mL. For each EOC dilution, bacteria were added to an inoculum concentration of approximately 5 $\log_{10}$ and were incubated for 48 h at 46 °C. Studies were individually carried out for each *A. acidoterrestis* strain.

For the MBC quantification, 1 mL of the samples whose absorbance did not increase was serially diluted 10-fold in peptone water and plated on BAT agar. Colonies were counted after 96 h of incubation at 46 °C. The results were expressed as $\log_{10}$ CFU/mL of *A. acidoterrestris.* Positive controls (bacteria in BAT broth and in BAT broth with 2.5% DMSO) and

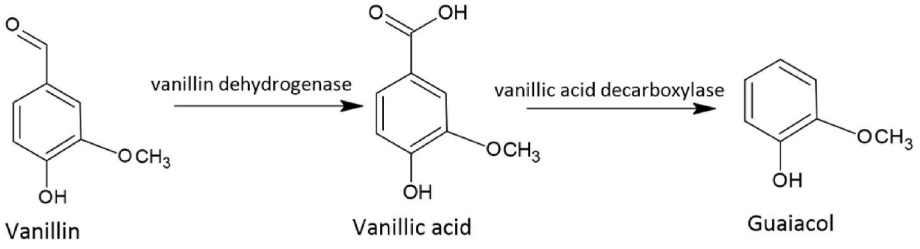

Fig. 1. Synthesis of guaiacol by *A. acidoterrestris* (Cai et al., 2015).

negative controls (BAT broth and BAT broth with 2.5% DMSO) were also included. The reported findings represent the mean of three independent repetitions performed in triplicate (n = 9).

2.3. Synthesis and characterisation of the EOC-functionalised silica particles

After selecting the most effective EOCs, commercial silica particles (**SiO_2**) of two particle size ranges (5–15 μm or 50–110 μm), employed as inorganic supports, were functionalised with the compounds in a two-step procedure. Firstly, silica particles (1g of 5–15 μm or 10 g of 50–110 μm) were combined with 0.15M of 3-aminopropyltriethoxysilane (APTES) in acetonitrile. Then, the reaction was stirred for 24 h at room temperature (RT). Next, the solid phase was centrifuged (11,000 *g*), washed with distilled water until a neutral pH and dried in a vacuum for 24 h to yield **SiO_2-APTES**. Eu or Thy was anchored onto **SiO_2-APTES** following the Mannich reaction described by Bishoyi et al. (2021) with some modifications (Fig. 2). Typically during synthesis, the obtained particles were mixed with 0.02 M of formaldehyde and 0.075 M of Eu or Thy in ethanol in a round-bottomed flask. Afterwards, reactions were stirred for 24 h at 60 °C, the solvent was removed by centrifugation (11,000 *g*) and particles were washed with distilled water until the leaching of EOCs and formaldehyde was removed. Finally, solids were dried in a vacuum for 24 h to yield the corresponding **SiO_2-Eu** and **SiO_2-Thy** solids.

The possible leaching of EOCs and formaldehyde by the functionalised particles was routinely analysed by HPLC following the procedure described by Pérez-Esteve et al. (2016) and Nageswari et al. (2012), respectively. The analysis was conducted with a Hitachi LaChrom Elite HPLC system (Hitachi Ltd., Tokyo, Japan) equipped with an autosampler (model L-2200) and a UV detector (model L-2400), and employing a Scharlab KromaPhase 100 C18 column (150 × 4.6 mm i.d., 5 μm, 100 Å) with a C18 guard column (10 mm × 4.6 mm). The UV detection wavelengths for Eu, formaldehyde and Thy were set at 280 nm, 360 nm and 277 nm, respectively.

The characterisation of the **SiO_2** and EOCs-functionalised silica particles (**SiO_2-EOCs**) involved standard techniques, which were run to assess both morphology and degree of functionalisation. A morphological analysis was conducted by field emission scanning electron microscopy (FESEM) with a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) in the secondary electron mode. Degree of functionalisation (mg EOC/g SiO_2) was determined from the elemental analyses of C, H and N using a Vario EL III Element Analyzer (Elemental Analyses System GMHB, Langenselbold, Germany) (Ruiz-Rico et al., 2017).

2.4. Assessment of the effect of the proposed non-thermal treatment on A. acidoterrestris growth and guaiacol formation

2.4.1. Non-thermal treatment of orange juice with free and immobilised EOCs

The effect of the applications of the EOCs-functionalised silica particles was evaluated by two different approaches: i) contact of the antimicrobial (free EOCs, **SiO_2** (used as a control) or **SiO_2-EOCs**) with the microorganism during a certain time period following the procedure reported by Gómez-Llorente et al. (2024b); ii) filtering juice through particles (**SiO_2** (control) or **SiO_2-EOCs**) as in the methodology proposed by Peña-Gómez et al. (2019a) or by Ruiz-Rico et al. (2021).

In the first approach, 10 mL of orange juice inoculated with *A. acidoterrestris* (5 $\log_{10}$) were incubated with MIC × 0.1, MIC × 0.25, MIC × 0.5 and MIC × 1 of the free EOCs and the equivalent concentrations of **SiO_2-EOCs** (5–25 μm) at 46 °C for 24 h with agitation (1000 *g*) to ensure uniform dispersion of the particles in the solution. The concentrations of the immobilised EOCs (**SiO_2-EOCs**), the equivalent to the free EOCs, were calculated from the degree of functionalisation as previously reported (Ruiz-Rico et al., 2017). At the highest particle concentration applied in the study, the effect of **SiO_2** was also included as a positive control. Studies were individually carried out for each *A. acidoterrestis* strain.

For the filtration treatment, a stainless-steel manifold filtration system (Microfil®, Merck Millipore, Darmstadt, Germany), connected to an Erlenmeyer flask for sample collection, was employed (Gómez-Llorente et al., 2024a). The collector assembly comprised two layers: first, a 3 cm layer of 50–110 μm particles (40 g), of approximately 26 cm^3 in volume, followed by cellulose paper. This study was done using 100 mL of orange juice inoculated (5 $\log_{10}$) with the strain that exhibited the greatest resistance in the previous treatment. The effect of **SiO_2** was also included as a positive control.

For both approaches, orange juice was supplemented with 100 mg/L of Va. This Va concentration proved optimal for the subsequent HPLC detection of the possible guaiacol formed by the microorganism (Shang et al., 2023).

2.4.2. Enumeration of vegetative cells of A. acidoterrestris

Vegetative cells of *A. acidoterrestris* after both treatments were quantified by the plate count technique on BAT agar after 96 h of incubation at 46 °C. To do so, each sample was 10-fold diluted in peptone water and bacterial colonies were enumerated. The results were expressed as $\log_{10}$ CFU/mL. All the treatments were done in triplicate (n = 3).

2.4.3. Determination of guaiacol formation

The effect of each treatment on guaiacol formation by the microorganism in orange juice was studied after 96 h. The temperature was fixed

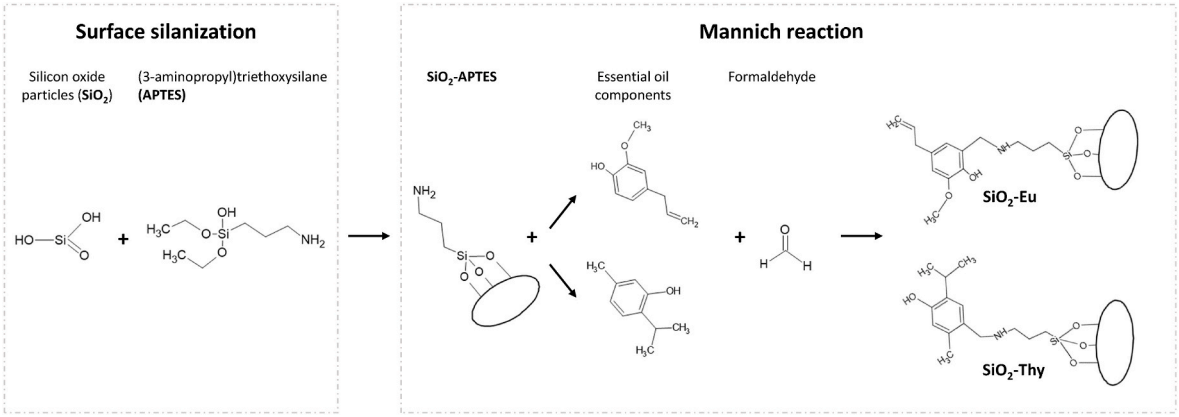

Fig. 2. Mannich reaction scheme and reagents for the immobilisation of eugenol (Eu) and thymol (Thy) on silica particles.

at 46 °C as is the optimal metabolic pathway temperature for guaiacol production by *A. acidoterrestris* (Savaş Bahçeci et al., 2005). For this purpose, the HPLC method described by Savaş Bahçeci et al. (2005) was employed with minor changes. The analysis was conducted using a Hitachi LaChrom Elite HPLC system (Hitachi Ltd., Tokyo, Japan) equipped with an autosampler (model L-2200) and a UV detector (model L-2400). A Scharlab KromaPhase 100 C18 column (150 × 4.6 mm i.d., 5 μm, 100 Å) with a C18 guard column (10 mm × 4.6 mm) was employed. The mobile phase, which consisted of 66.5 % deionised water, 3.5% formic acid and 30% methanol for 15 min, was used in the isocratic mode at a flow rate of 1.0 mL/min at 25 °C, and with an injection volume of 10 μL. The UV detection wavelength was set at 350 nm. HPLC analyses were conducted in triplicate (n = 3).

2.5. Statistical analysis

After each treatment, the bacterial growth (log_{10}) and guaiacol production (mg/L) was statistically analysed by a one-way ANOVA with a 95% confidence interval ($p < 0.05$) using Statgraphics Centurion XVIII (Statpoint Technologies, Inc., Warrenton, VA, USA). A Multifactor ANOVA was employed to determine the effect of each variable (EOC type, EOC concentration, dosage form and strain) on bacterial growth (log_{10}) and guaiacol production (mg/L).

3. Results and discussion

3.1. In vitro susceptibility of A. acidoterrestris strains to the different EOCs

In the initial phase of our study against *A. acidoterrestris*, four different EOCs were strategically chosen for their different chemical compositions and documented efficacy in combating spoilage microorganisms in food-related applications (Nourbakhsh et al., 2022; Perricone et al., 2015). By incorporating EOCs with diverse functional groups and established antimicrobial properties, our study aimed to explore a series of solutions based on non-thermal treatments to mitigate the alteration of food product quality. Table 1 shows the MIC and the MBC of Eu, Ger, Thy and Va against different *A. acidoterrestris* strains.

An analysis of the MIC revealed significant variations among the types of EOCs, attributable to differences in molecular structure and the investigated bacterial strains ($p < 0.001$). Thy had the greatest antimicrobial activity of all the tested compounds, with MIC values ranging from 0.75 to 2.5 mg/mL depending on the studied strain. In line with this evaluation, Cai et al. (2019) reported a MIC of 0.25 mg/mL when assessing the impact of Thy on the growth of *A. acidoterrestris*. Discrepancies between these results and our work could be possibly attributed to the strain used in their study (*A. acidoterrestris*, DSM 3924). Eu demonstrated notable antimicrobial activity against the different *A. acidoterrestris* strains, with MICs of 3.75 mg/mL for four of the five strains, and of 5 mg/mL for the most resistant one. Although the literature in this case is scarce, our results agree with those of Bevilacqua et al. (2010), who evaluated the antimicrobial activity of Eu against *A. acidoterrestris*. Specifically, 0.08 mg/mL Eu was found to be ineffective in reducing bacterial growth. For Ger, no antimicrobial activity was observed against any of the evaluated strains, which would explain the lack of relevant studies on this EOC. Finally, Va exhibited moderate antimicrobial activity, with a MIC of 3.75 mg/mL against one of the strains and one of 5 mg/mL for the remaining strains.

Despite applying very high concentrations (<10 mg/mL), the MBC was not reached for any of the EOC-strains combinations. Higher concentrations than 10 mg/mL were not tested because it exceeded the solubility limit of EOCs with selective media. Not obtaining MBC findings is consistent with this microorganism's significant resistance to treatments (Pornpukdeewattana et al., 2020; Silva et al., 2015). Considering the results obtained after determining the MIC and MBC of Eu, Ger, Thy and Va *in vitro* against five different *A. acidoterrestris* strains, Eu and Thy were selected for our next study phases.

Table 1
The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of eugenol (Eu), geraniol (Ger), thymol (Thy) and vanillin (Va) against the different *Alicyclobacillus acidoterrestris* strains. The results are expressed as mg/mL. Mean value ± SD (n = 3).

Strain	Eu		Ger		Thy		Va	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
St1	3.75^{aB}	>10	>10	>10	2.5^{cA}	>10	5^{bC}	>10
St2	5^{bB}	>10	>10	>10	1^{bA}	>10	5^{bB}	>10
St3	3.75^{aB}	>10	10^{D}	>10	0.75^{aA}	>10	5^{bC}	>10
St4	3.75^{aB}	>10	10^{D}	>10	1^{bA}	>10	5^{bC}	>10
St5	3.75^{aB}	>10	>10	>10	0.75^{aA}	>10	3.75^{aB}	>10

*Different lower case letters in the same column indicate significant differences in the MIC and MBC values among strains. Different capital letters in the same row denote significant differences among treatments ($p < 0.001$).

3.2. In vivo effect of incorporating free eugenol and thymol on the bacterial growth and guaiacol production of A. acidoterrestris in orange juice

After evaluating the *in vitro* antimicrobial activity of EOCs, the effect of free Eu and Thy on antimicrobial activity and the inhibition of guaiacol production was assessed with commercial orange juice for 24 h at 46 °C. The antimicrobial concentrations selected for conducting these experiments were set at MIC × 0.1, MIC × 0.25, MIC × 0.5 and MIC × 1 for each evaluated strain (more details appear in Section 2.3.3).

In relation to the antimicrobial activity of the free natural compounds, Fig. 3 shows the effect of the various treatments with free Eu and Thy on the growth of the different *A. acidoterrestris* strains. As it depicts, all the strains had a similar growth, approximately 5 log_{10} CFU/mL, when antimicrobial treatment was lacking ($p > 0.05$).

Regarding the free Eu application, Fig. 3A shows that the application of MIC × 0.1 did not impact bacterial growth because similar counts to the control were found ($p > 0.05$). At MIC × 0.25 of Eu, bacterial growth significantly reduced in three of the five evaluated strains, but the same treatment showed no significant effects on strains St3 and St5 ($p > 0.05$). When increasing the Eu concentration to MIC × 0.5 and MIC × 1, bacterial growth approximately reduced by 4 log_{10} cycles. Despite the observed reduction at the highest antimicrobial concentration, two distinct groups of strains were statistically significant: the most resistant (St3 and St5) and the most sensitive (St1, St2, and St4) strains.

With Thy (Fig. 3B), applying MICx0.1 or MIC × 0.25 did not lead to any major reduction in any of the evaluated strains. Increasing the concentration to MIC × 0.5 led to bacterial reduction of ca. 3.5 log_{10} cycles. Unlike the observations made with Eu, doubling the Thy concentration from MIC × 0.5 to MIC × 1 resulted in a significant reduction in only one of the five evaluated strains. Notably at MIC × 1, the resistance of strains St3 and St5 was greater. The significant reduction in the different strains when values below the MIC were applied suggests that orange juice has compounds, such as organic acids (i.e. citric acid) that would favour the effect of EOCs (Gómez-Llorente et al., 2024a).

In parallel to the study into the effect of natural antimicrobials on reducing bacterial growth, the effect on guaiacol formation inhibition by the different *A. acidoterrestris* strains was also investigated. Table 2 shows the guaiacol concentration in orange juice after performing incubation with free Eu and Thy. Remarkably, the treatments with both the free natural antimicrobials at MICx0.5 and MICx1 effectively suppressed guaiacol production to undetectable levels for the different microorganism strains. The treatment run with MIC × 0.25 of Eu completely inhibited guaiacol production in strains St1, St2 and St4, whereas MIC × 0.5 was necessary to inhibit guaiacol formation with strains St3 and St5. With Thy, all the different strains inhibited guaiacol formation at the same concentration (MIC × 0.5).

The effect of Thy on guaiacol production by *A. acidoterrestris* DSM

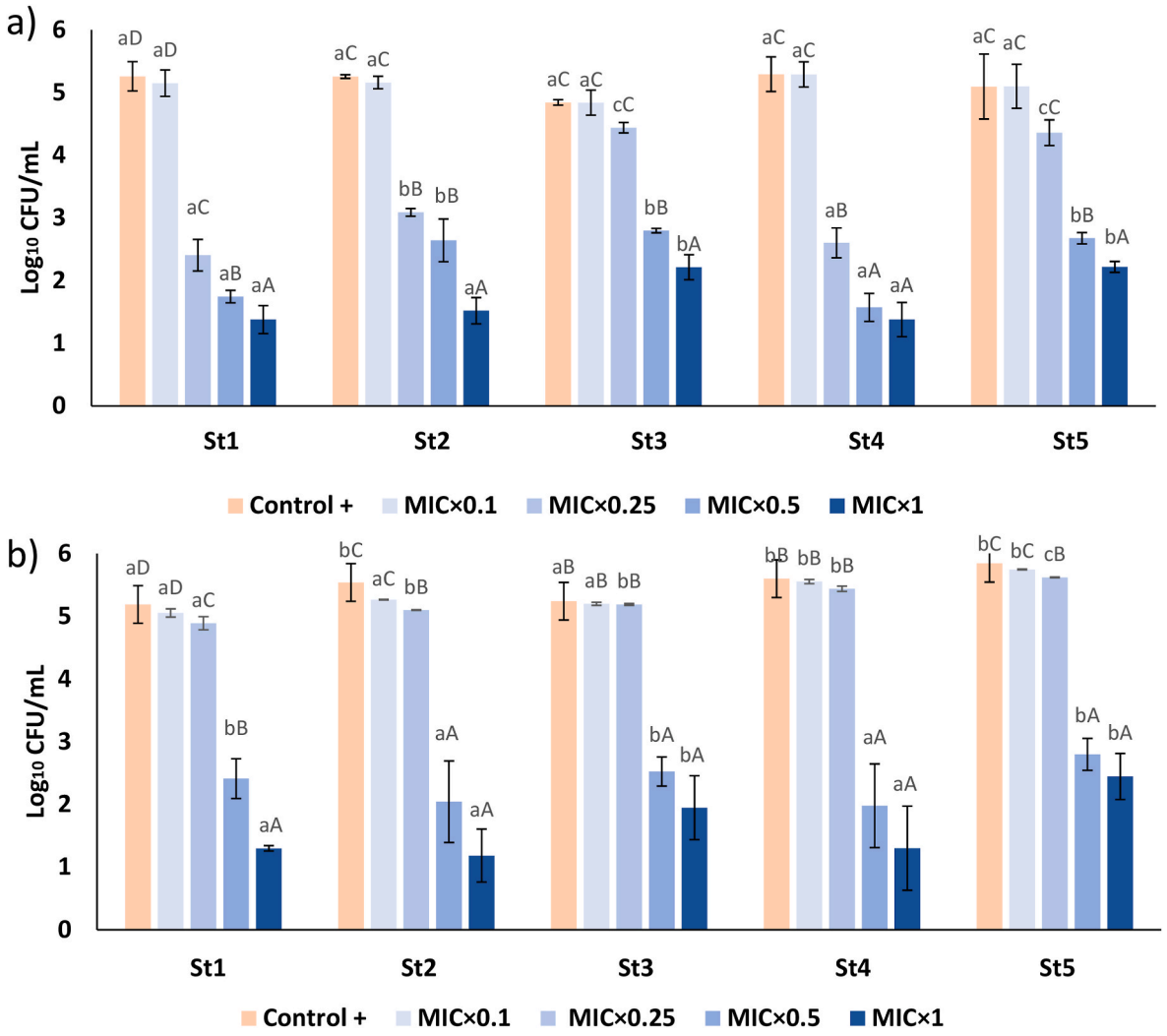

Fig. 3. Effect of incorporating free a) Eu and b) Thy at MIC × 0.1, MIC × 0.25, MIC × 0.5 and MIC × 1 in orange juice on the growth of the different A. *acidoterrestris* strains (St1, St2, St3, St4, St5). Mean value ± SD (n = 3). Different small letters indicate significant differences in the *A. acidoterrestris* counts among strains; different capital letters denote statistically significant differences in the *A. acidoterrestris* counts among treatments *($p < 0.05$)*.

Table 2
Effect of the different treatments of free Eu and Thy at MIC × 0.1, MIC × 0.25, MIC × 0.5 and MIC × 1 on the production of guaiacol. The results are expressed as mg/L of guaiacol. Mean values (3) ± SD.

Strain	EOC	Control	MIC × 0.1	MIC × 0.25	MIC × 0.5	MIC × 1
St1	Eu	77 ± 3^{bB}	76 ± 3^{bB}	$n.d^{aA}$	$n.d^{aA}$	$n.d^{aA}$
St2		61.9 ± 1.3^{aB}	58 ± 3^{aB}	$n.d^{aA}$	$n.d^{aA}$	$n.d^{aA}$
St3		95 ± 5^{cC}	92 ± 4^{cC}	24 ± 4^{bB}	$n.d^{aA}$	$n.d^{aA}$
St4		84 ± 5^{bcB}	86 ± 7^{bcB}	$n.d^{aA}$	$n.d^{aA}$	$n.d^{aA}$
St5		97.2 ± 0.9^{cC}	95 ± 5^{cC}	51 ± 2^{cB}	$n.d^{aA}$	$n.d^{aA}$
St1	Thy	74.6 ± 1.1^{bC}	71 ± 2^{bB}	70 ± 3^{cB}	$n.d^{aA}$	$n.d^{aA}$
St2		63 ± 3^{aC}	65 ± 4^{aC}	52 ± 3^{aB}	$n.d^{aA}$	$n.d^{aA}$
St3		90 ± 7^{cC}	92 ± 3^{cC}	60 ± 2^{bB}	$n.d^{aA}$	$n.d^{aA}$
St4		87 ± 3^{cC}	88 ± 5^{cC}	61 ± 2^{bB}	$n.d^{aA}$	$n.d^{aA}$
St5		95 ± 4^{cB}	91 ± 3^{cB}	90 ± 2^{dB}	$n.d^{aA}$	$n.d^{aA}$

* Different lower case letters in the same column indicate significant differences in the guaiacol concentration among strains; different capital letters in the same row denote statistically significant differences in the guaiacol concentration among treatments ($p < 0.001$).

3924 has been evaluated in broth media by Cai et al. (2019), who found that 0.25 mg/mL of Thy totally inhibited guaiacol production by the microorganism. In our study, higher Thy concentrations (0.375 mg/mL for strains St3 and St5; 0.5 mg/mL for strains St2 and St4; 1.25 mg/mL for strain St1 were required to achieve a similar effect. These differences could be attributed to the fact that the guaiacol production rate depends on the specific strain (Van Luong et al., 2019) or could be due to the different sensitivities among the EOC-strains combinations (Mansouri et al., 2018).

Finally, the relation between antimicrobial activity (Fig. 3) and guaiacol inhibition (Table 2) for the treatments with free Eu and Thy was analysed. At MIC × 0.5, both antimicrobials effectively inhibited guaiacol formation, but the viable bacterial counts suggest two hypotheses. As stated by Shang et al. (2023), the inhibition of the guaiacol concentration would depend on microorganism growth. What this implies in our study is that the bacterial population required to produce a detectable guaiacol level might be higher than 2.5 $\log_{10}$. Alternatively, guaiacol production inhibition would be due to a combination of effects that go beyond microorganism growth because EOCs could cause sublethal damages or lead to conformational or structural alterations in enzymes, such as dehydrogenase or decarboxylase involved in guaiacol metabolic pathways (Cai et al., 2015; Wahia et al., 2022).

3.3. *In vivo effect of incorporating immobilised eugenol and thymol on the bacterial growth and guaiacol production of A. acidoterrestris in orange juice*

After confirming the capability of free Eu and Thy to reduce bacterial growth and to inhibit guaiacol production, the next step was to assess if this reduction could be achieved using the EOCs immobilised onto silica microparticles. For that purpose, the silica particles with two different particle sizes (5–15 μm for the contact studies or 50–110 μm for the filtration studies) were functionalised with Eu and Thy.

3.3.1. *Characterisation of the functionalised silica particles*

The functionalised silica particles were characterised using FESEM and an elemental analysis to corroborate the efficiency of the functionalisation procedure based on the Mannich reaction. Fig. 4 illustrates the morphology of **SiO_2**, **SiO_2-Eu** and **SiO_2-Thy**, which ranged from 5 to 15 μm in the left row, and was 50–110 μm in the right row. No discernible difference in the support surface was detected when comparing **SiO_2** to **SiO_2-EOCs**, which confirmed that the immobilisation process did not impact the support's integrity. These results agree with the previous findings reported by García-Ríos et al. (2018), Gómez-Llorente et al. (2024b), Peña-Gómez et al. (2019b), Peña-Gómez et al. (2020) and Ruiz-Rico et al. (2021), where Eu and Thy were covalently anchored onto the surface of silica microparticles. Moreover, the particle sizes of both particle types matched their technical specifications and did not exhibit any variations after functionalisation.

An elemental analysis was employed to quantify the amount of EOCs attached to the surfaces of silica particles. The elemental analysis results showed that **SiO_2-EOCs** possessed approximately 100.79 and 95.34 mg EOC/g SiO_2 for Eu, and 64.62 and 67.71 mg EOC/g SiO_2 for Thy, on supports, within the 5–15 μm range and the 50–110 μm range, respectively. The degree of functionalisation results indicated that the **SiO_2-EOCs** used in this work were properly functionalised.

3.3.2. *Treatment based on adding functionalised particles to juice*

The first approach to employ the functionalised silica particles to control bacterial growth and guaiacol production by *A. acidoterrestris* consisted of adding different amounts of 5–15 μm **SiO_2-EOCs** to juice. To carry out these experiments, the equivalent concentrations of **SiO_2-EOCs** to those of the free Eu and Thy forms were employed, and they were stirred for 24 h. Fig. 5 shows the growth of the different strains after incubation with **SiO_2** and **SiO_2-EOCs**. It depicts how **SiO_2** did not affect microorganism growth ($p > 0.05$), and the same counts were obtained as in the control (no particles). Lack of antimicrobial activity by bare silica particles has been previously reported by Peña-Gómez et al. (2019b) against *Escherichia coli*. In contrast, the immobilisation of the different EOCs onto silica particles reduced bacterial growth, albeit with differences between antimicrobials and strains. The treatments with **SiO_2-Eu** slightly diminished the microorganism when applying a concentration of MIC × 0.25 for strains St1 and St2 compared to the application of MIC × 0.1 or **SiO_2** ($p < 0.05$). Doubling the concentration to MIC × 0.5 led to bacterial reduction of ca. 4 $\log_{10}$, which was more than 99.9% of the bacterial counts. The complete reduction of strains St1 and St2 (>4.5 $\log_{10}$) occurred when MIC × 1 was applied. For the

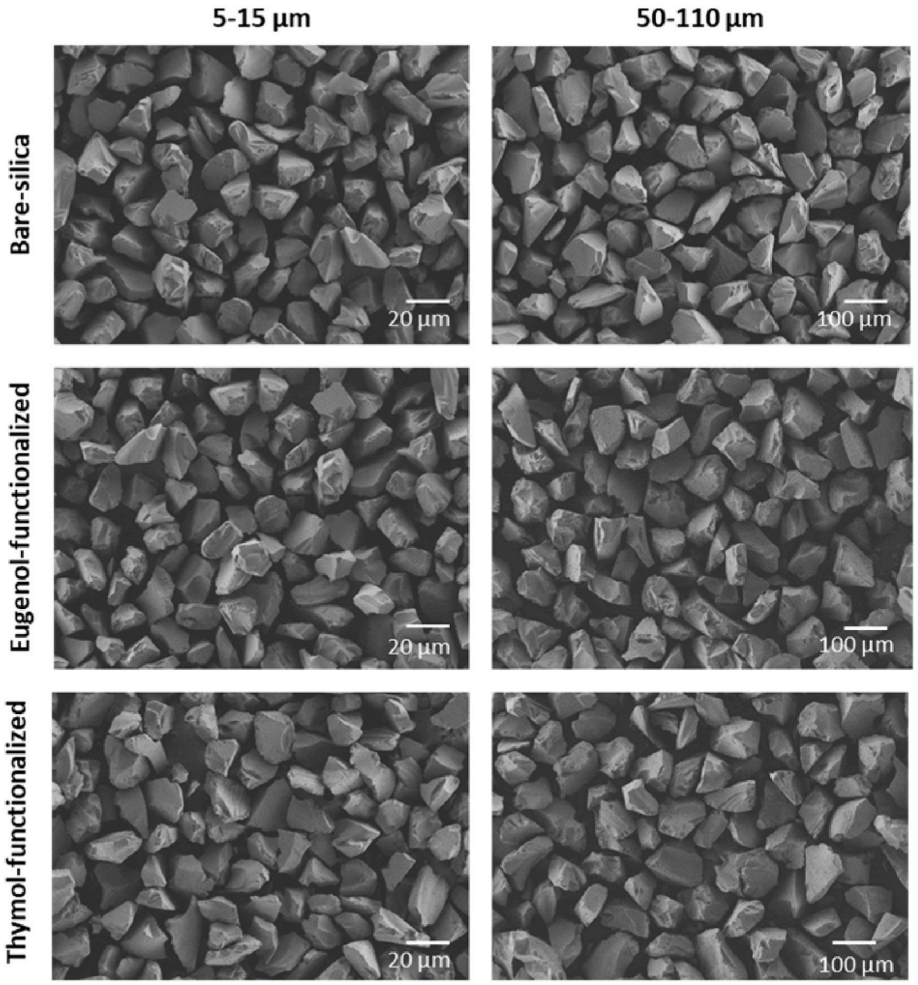

Fig. 4. FESEM images of 5–15 μm (left column) 50–110 μm (right column) for both the bare (**SiO_2**) and silica particles functionalised with eugenol (**SiO_2-Eu**) and thymol (**SiO_2-Thy**).

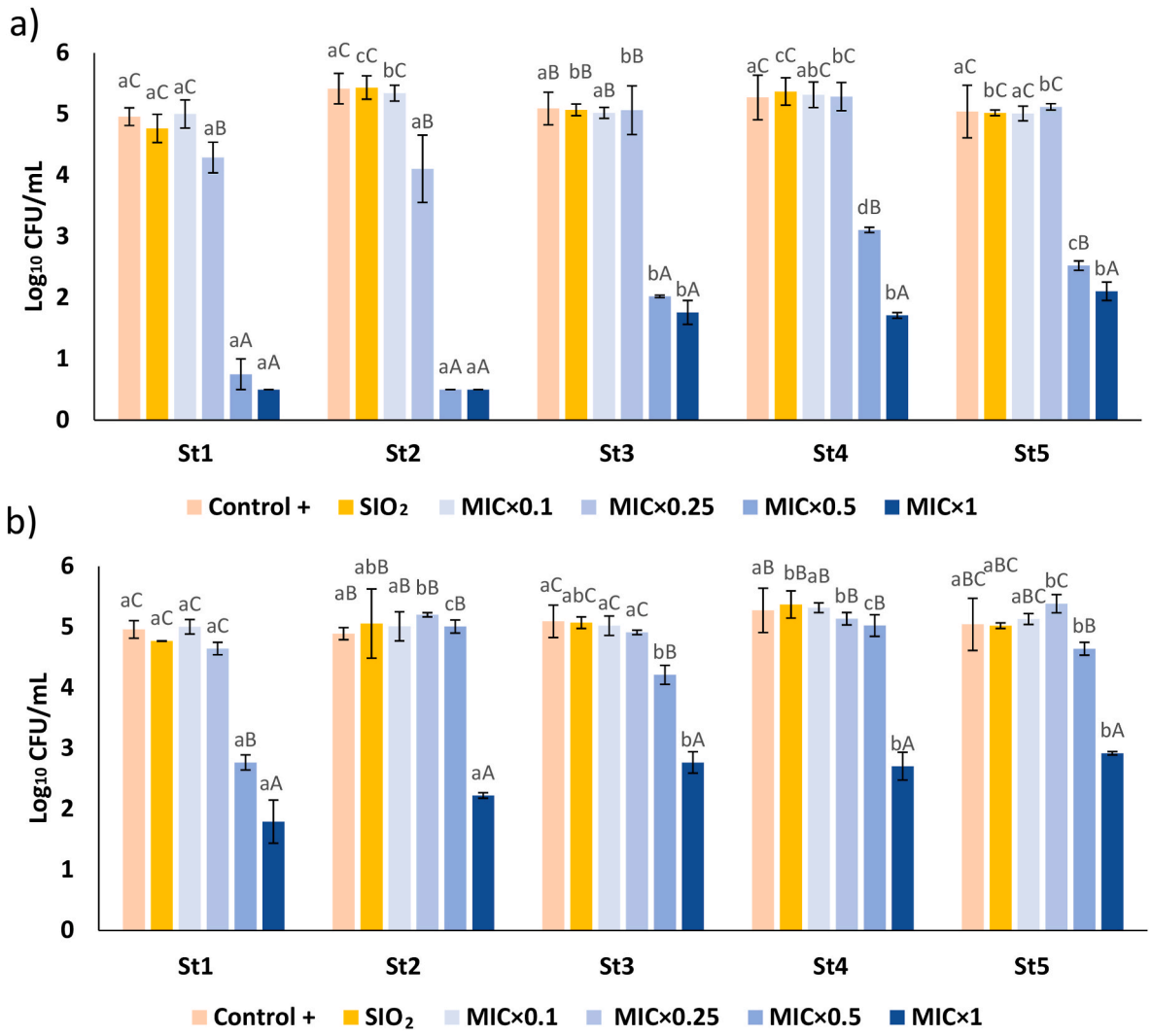

Fig. 5. Effect of incorporating a) **SiO_2-Eu** and b) **SiO_2-Thy** at MIC × 0.1, MIC × 0.25, MIC × 0.5, MIC × 1 in orange juice on the growth of the different A. *acidoterrestris* strains (St1, St2, St3, St4, St5). Mean value ± SD (n = 3). Different small letters indicate significant differences in the *A. acidoterrestris* counts among strains; different capital letters denote statistically significant differences in the *A. acidoterrestris* counts among treatments *($p<0.05$)*.

treatments with **SiO_2-Thy**, a significant reduction of approximately 3 log_{10} was observed after applying MIC × 1 of functionalised particles. These findings indicate less antimicrobial activity for **SiO_2-Thy** compared to **SiO_2-Eu**.

Despite studies not having specifically focused on evaluating the antimicrobial properties of immobilised EOCs against *A. acidoterrestris*, several research works have explored their antimicrobial efficacy in fruit juice. For instance, Ribes et al. (2019) examined the effect of silica particles functionalised with Eu against *E. coli* in both apple juice and grape juice. These authors observed that the treatments with 0.05 mg/mL and 0.125 mg/mL of immobilised Eu, which lasted 2 h, resulted in effective *E. coli* reduction (below the limit of detection) in apple juice and grape juice, respectively. Liu et al. (2022) investigated the inhibitory effect of Thy-functionalised onto silica particles and found that 0.2 mg/mL of particles inhibited *E. coli* growth in apple juice. The requirement for higher concentrations than those reported against other bacteria would indicate the robustness of *A. acidoterrestris* to treatments. Despite this challenge, using EOCs as a non-thermal treatment underscores the potential of such strategies for combating *A. acidoterrestris* contamination. Non-thermal treatments offer distinct advantages in food preservation, such as preserving sensory attributes and nutritional value of food products, while also mitigating the risk of detrimental effects associated with heat treatments, such as nutrient loss and undesirable changes in texture or flavour (Gómez-Llorente et al., 2023).

When comparing the effects of the free and immobilised EOCs on the growth of different strains (see Figs. 3 and 5 respectively), distinct patterns emerged. Firstly, both the free and immobilised forms of EOCs resulted in significant reductions when applying MIC × 0.5 of the antimicrobial. Secondly, of all the treatments, applying MIC × 1 of **SiO_2-Eu** achieved the most marked reduction in microorganism growth. This suggests that the immobilisation of EOCs enhances their activity, possibly due to the higher local concentration of the antimicrobial achieved after being immobilised on silica surfaces (Peña-Gómez et al., 2018).

Table 3 shows data about the capability of **SiO_2** and **SiO_2-EOCs** to inhibit guaiacol production in orange juice. As expected, **SiO_2** did not inhibit guaiacol production in any of the evaluated strains because antimicrobial activity was lacking ($p > 0.05$). On the contrary, the treatment with the functionalised strains reduced the production of this undesired metabolite; the higher the antimicrobial concentration, the greater guaiacol inhibition was ($p < 0.001$). Applying a concentration of MIC × 0.5 or MIC × 1 led to completely inhibited guaiacol production for both the tested antimicrobials. Despite complete guaiacol inhibition, the same antimicrobial concentration did not completely reduce microorganism growth (see the details in Fig. 5), which suggests that sublethal concentrations of EOCs would inhibit guaiacol production by

Table 3
Effect of incorporating **SiO_2-Eu** and **SiO_2-Thy** at MIC × 0.1, MIC × 0.25, MIC × 0.5 and MIC × 1 in orange juice on guaiacol production. The results are expressed as mg/L of guaiacol. Mean values (3) ± SD.

Strain	EOC	Control	SiO_2	MIC × 0.1	MIC × 0.25	MIC × 0.5	MIC × 1
St1	Eu	77 ± 6^{bD}	77 ± 4^{bD}	70.7 ± 1.3^{bC}	49 ± 3^{bB}	$n.d^{aA}$	$n.d^{aA}$
St2		60 ± 7^{aD}	60 ± 3^{aD}	55 ± 0.6^{aC}	35 ± 8^{aB}	$n.d^{aA}$	$n.d^{aA}$
St3		92 ± 3^{cD}	91 ± 3^{cD}	78 ± 6^{cC}	58 ± 6^{bcB}	$n.d^{aA}$	$n.d^{aA}$
St4		81 ± 6^{bcC}	80 ± 6^{bC}	68 ± 5^{bB}	62 ± 5^{cB}	$n.d^{aA}$	$n.d^{aA}$
St5		93 ± 5^{cD}	92 ± 2^{cD}	73 ± 5^{bcC}	54.4 ± 0.9^{bB}	$n.d^{aA}$	$n.d^{aA}$
St1	Thy	76 ± 3^{bC}	73 ± 5^{bBC}	72 ± 2^{bB}	70 ± 2^{cB}	$n.d^{aA}$	$n.d^{aA}$
St2		67 ± 4^{aC}	68 ± 4^{aC}	61 ± 3^{aC}	53 ± 5^{aB}	$n.d^{aA}$	$n.d^{aA}$
St3		92 ± 2^{dD}	90 ± 2^{cD}	84 ± 5^{cC}	64 ± 2^{bB}	$n.d^{aA}$	$n.d^{aA}$
St4		84 ± 2^{cD}	84 ± 4^{bcD}	73 ± 4^{bC}	59 ± 5^{abB}	$n.d^{aA}$	$n.d^{aA}$
St5		95 ± 7^{dD}	90 ± 7^{cD}	83 ± 5^{cC}	68.0 ± 0.8^{cB}	$n.d^{aA}$	$n.d^{aA}$

Different lower case letters in the same column indicate significant differences in the guaiacol concentration among strains; different capital letters in the same row denote statistically significant differences in the guaiacol concentration among treatments ($p < 0.001$).

the different *A. acidoterrestris* strains. When analysing data at the time when guaiacol was still detectable, **SiO_2-Eu** achieved greater guaiacol inhibition in most strains than **SiO_2-Thy** at the same particle concentration.

To compare the effect of the strain, the type of EOC, the concentration and the dose form of EOCs on the inhibition of both microbial growth (log_{10}) and guaiacol production (mg/mL), a multifactorial statistical analysis was conducted. As shown in Table S1, all the factors except for the dose form (free or immobilised) had a significant effect on both microbial growth and guaiacol inhibition. These results are very interesting because the immobilisation process maintained the antimicrobial activity of the evaluated EOCs, while offering significant advantages, such as a slighter impact on the sensory profile (Ribes et al., 2017), easy dosing, and the possibility of removing active ingredients by centrifugation or filtration, and then reusing them in a subsequent batch.

3.4. Treatment based on juice filtration though functionalised particles

Having demonstrated that the immobilised EOCs effectively reduced growth and inhibited guaiacol production in *A. acidoterrestris* by coming into contact with agitation, which involved a 24-h incubation period, we further investigated the potential of **SiO_2-Eu** and **SiO_2-Thy** as filtering aids (García-Ríos et al., 2018; Peña-Gómez et al., 2019a). During this approach, a 100 mL volume of inoculated orange juice with strain St5 was filtered through 40 g of **SiO_2-Eu** and **SiO_2-Thy** in less than 1 min. Strain selection was based on its high guaiacol production and its greater resistance to antimicrobial treatments, as evidenced in Table 3 and Fig. 5, respectively.

Table 4 shows the bacterial counts and guaiacol production by the microorganism after filtering inoculated juice through a layer of **SiO_2** and **SiO_2-EOCs**. As illustrated, filtration though the bare silica particles had no influence on bacterial counts. In contrast, filtration through **SiO_2-EOCs** significantly influenced bacterial removal ($p < 0.001$). The treatments with **SiO_2-Eu** led to a reduction that exceeded 4.3 log_{10}, whereas **SiO_2-Thy** resulted in a reduction of 1.4 log_{10}.

The antimicrobial activity of the functionalised silica particles with

Table 4
Effect of filtering orange juices through **SiO_2-Eu** and **SiO_2-Thy** on *A. acidoterrestris* counts and guaiacol production. The results are expressed as log_{10} and mg/L of guaiacol for removal capability and guaiacol production, respectively. Mean values (3) ± SD.

	Bacteria counts (log_{10})	Guaiacol (mg/L)
Control	5.0 ± 0.3^{C}	95.83 ± 6.02^{B}
SiO_2	4.8 ± 0.2^{C}	94.37 ± 5^{B}
Eu	0.50 ± 0.00^{A}	$n.d^{A}$
Thy	3.4 ± 0.5^{B}	$n.d^{A}$

* Different capital letters in the same column denote statistically significant differences in bacteria counts or guaiacol concentrations among treatments *($p < 0.05$)*.

Eu and Thy across various microorganisms and food media has been previously explored. Peña-Gómez et al. (2019a) demonstrated a significant reduction of 4 log_{10} in *E. coli* in apple juice filtered through Eu-functionalised silica particles, along with complete indigenous juice microbiota removal, including mesophilic, psychrophilic bacteria and mould/yeast. This resulted in the microbial stabilisation of juice on the first 120 refrigerated storage days. Peña-Gómez et al. (2020) also examined the effect of immobilised Eu and Thy on *E. coli* removal in craft beer. They reported a reduction of approximately 2.5 log_{10}, the equivalent to more than 99% of total bacterial counts. Ruiz-Rico et al. (2021) assessed the efficacy of immobilised Eu in reducing common microorganisms in wines, such as bacteria (*Acetobacter aceti* and *Lactobacillus plantarum*), fungi (*Dekkera bruxellensis* and *Zygosaccharomyces bailii*) and yeast (*Saccharomyces cerevisiae*). Their findings indicated a 95% count reduction for most of the tested microorganisms, which is comparable to alternative treatments that involve pulsed electric fields or high hydrostatic pressure. The variation in removal capability reported by these authors and our work could be attributed to a combination of factors, including the employed specific microorganism strains and the food matrix composition (Gómez-Llorente et al., 2024a; Hyldgaard et al., 2012).

Table 4 also indicates that passing **SiO_2** through a filter did not significantly inhibit guaiacol production because a similar concentration compared to lack of particles was observed ($p > 0.05$). On the contrary, the treatments with the immobilised EOCs brought about complete guaiacol production inhibition by *A. acidoterrestris* strain 5. Bearing this in mind, if the orange juice filtration treatment inhibited the most resistant strain and was that which produced the largest amount of guaiacol (see the details in Section 3.4), it is reasonable to assume that similar inhibition would occur in the remaining strains.

Considering the aforementioned findings, the effect of filtering inoculated orange juice through **SiO_2-EOC** on guaiacol production inhibition could be proposed as a feasible non-thermal treatment in juice industries. This is an interesting preservation strategy to avoid sensory alterations due to guaiacol formation and to, therefore, reduce waste and economic losses (Shang et al., 2023). Furthermore, the immobilisation of EOCs onto silica surfaces would offer a viable solution to address solubility issues and the undesirable odours or flavours that result from EOCs applications (Ribes et al., 2019).

It is necessary to state that the filtering technology could be scaled up in a real scenario (Peña-Gómez et al., 2019a). Indeed, utilizing this technology as processing aids would offer distinct advantages over its application as additives. For instance, Kwon et al. (2023) indicated that the regulatory process for new materials that act as processing aids is generally simpler across most evaluated countries when reviewing food additive and processing aid regulations. It is also indicated that consumer perceptions are more favourable towards new technologies when they are used as processing aids (Gómez-Llorente et al., 2022).

4. Conclusions

The present study offers novel insights into the efficacy of two

different approaches that involve Eu and Thy immobilised on silica particles to reduce vegetative cells of *A. acidoterrestris* growth and to inhibit guaiacol production in orange juice. The results reveal that the immobilisation process does not alter the antimicrobial concentration required to inhibit guaiacol production by microorganisms, which remained at MIC × 0.5 for all the evaluated strains. This finding is particularly noteworthy because the immobilisation process preserves the antimicrobial effectiveness of EOCs, while avoiding product alteration by the presence of guaiacol. The application of this technology would also avoid the main problems related to free EOCs applications, such as alterations to food products' odour and taste. Finally, shorter particle contact by the filtration process through the Eu- and Thy-functionalised particles bed also results in completely inhibited guaiacol production.

Considering the reduction in bacterial counts and the guaiacol inhibition results obtained with both the proposed approaches, coupled with the GRAS status of the studied EOCs and silica particles, our study underscores the prospective industrial applicability of non-thermal treatment during the juice production process. So, the food industry could effectively meet consumer demand for safe, high-quality products, while making the most of the inherent benefits of natural compounds. It could also reinforce its commitment to offer products free of sensory alterations and could, thereby, address the current serious food waste problem.

CRediT authorship contribution statement

Héctor Gómez-Llorente: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Oumaima Moumane:** Writing – original draft, Formal analysis, Data curation. **Sergio Grau-Martínez:** Investigation, Formal analysis, Data curation. **Ana Isabel Jiménez-Belenguer:** Supervision, Methodology. **Manuel Hernández:** Supervision, Methodology. **María Ruiz-Rico:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **José M. Barat:** Methodology, Funding acquisition. **Isabel Fernández-Segovia:** Writing – review & editing, Validation, Supervision, Methodology, Data curation. **Édgar Pérez-Esteve:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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