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New strategies to enhance the quality and safety of liquid foods based on the use of natural antimicrobial compounds

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CERTIFY:

That the work *"***New strategies to enhance the quality and safety of liquid foods based on the use of natural antimicrobial compounds"** has been developed by Héctor Gómez Llorente under their supervision in the Instituto Universitario de Ingeniería De Alimentos FoodUPV at the *Universitat Politècnica de València*, as a Thesis Project in order to obtain the degree of PhD in Food Science, Technology and Management at the *Universitat Politècnica de València.*

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Abstract

Essential oil components (EOCs) have proven to be effective against a wide variety of microorganisms. However, the direct application of these compounds poses challenges due to their low solubility and alteration of the organoleptic properties of foods. In response to these limitations, the search for new dosage forms of these promising antimicrobials is essential to promote their use in the food industry.

The present doctoral thesis is focused on the development and application of antibacterial and antiviral systems based on the covalent immobilization of EOCs to improve the quality and safety of liquid foods.

The first chapter evaluated the effect of the addition of components naturally present in food (proteins, lipids, carbohydrates, organic acids and ethanol) on the antimicrobial activity of certain EOCs (carvacrol, eugenol, geraniol, thymol and vanillin) in their free form. In this first part, the influence of these food components on the antimicrobial activity of vanillin immobilized on silicon oxide particles was also evaluated. The results showed that bovine serum albumin (BSA), sunflower oil and some carbohydrates were the food components that most inhibited totally or partially the antimicrobial activity of the free EOCs evaluated. However, some exceptions were found. In media containing BSA the antimicrobial activity of geraniol was not inhibited. The same occurred with eugenol in media containing sunflower oil, or with carvacrol, eugenol, geraniol and thymol in media containing D-lactose. Likewise, the study carried out on immobilized vanillin confirmed the inhibitory effect of the proteins, lipids and carbohydrates evaluated on the antimicrobial activity. However, other components such as citric acid and ethanol enhanced the antimicrobial activity, thus overcoming the hindering effect. These results demonstrate the importance of considering the composition of the food matrix when selecting an antimicrobial compound.

After having studied the effect of the different components naturally present in food, the second chapter evaluated the application of EOCs against the growth and production of guaiacol by a spoilage and heat-resistant microorganism such as

Abstract

Alyciclobacillus acidoterrestris in orange juice. For this purpose, EOCs immobilized on silicon oxide particles were used with two different approaches: as additives and as processing aids. The results showed that the presence of EOCs causes a microbial reduction and an inhibition of guaiacol production, which is maintained after immobilization of the antimicrobials. This fact is of great interest, since immobilization avoids the problem of organoleptic alteration of the product, derived from the application of these antimicrobials in free form.

In the third chapter, the antiviral activity of EOCs, both in free and immobilized form, against Tulane virus in water was studied. The results showed that, while the application of EOCs in free form achieved a reduction in infectivity of only 1 $\log_{10}$, equivalent concentrations of immobilized antimicrobial reduced infectivity to undetectable levels (reduction of more than 4.5 $\log_{10}$). On the other hand, it has been demonstrated that the antiviral mechanism is based on the ability of immobilized antimicrobials to modify or disrupt the viral capsid, which is responsible for giving the virus its infective properties. Furthermore, it was determined that immobilized EOCs are not cytotoxic at effective antiviral concentrations.

Despite the efficacy observed after immobilization of EOCs on silicon oxide particles against bacteria and viruses, their practical application in the food industry presents several challenges, one of them is the acceptance of this technology by consumers. For this reason, the last chapter studied the perception of consumers regarding the use of nanotechnology in food processing. The evaluation of the different foods in which nanotechnology had been applied in their processing or packaging was generally positive, and most consumers would buy them. Of all the products, those in which nanotechnology was not part of the food received the best evaluation. Considering this result, immobilized EOCs that are applied as processing aids would be the most highly valued, and therefore could be an excellent alternative to conventional preservation treatments, to control both viruses and bacteria during food production and storage.

Resumen

Los componentes de aceites esenciales (CAEs) han resultado ser efectivos frente a una gran variedad de microrganismos. No obstante, la aplicación directa de estos componentes plantea desafíos debido a su baja solubilidad y a la alteración de las propiedades organolépticas de los alimentos. En respuesta a estas limitaciones, la búsqueda de nuevas formas de dosificación de estos agentes tan prometedores es vital para favorecer su uso en la industria alimentaria.

La presente tesis doctoral está centrada en el desarrollo y aplicación de sistemas antibacterianos y antivirales basados en la inmovilización covalente de CAEs para mejorar la calidad y seguridad de los alimentos líquidos.

En el primer capítulo se evaluó el efecto de la adición de componentes naturalmente presentes en los alimentos (proteínas, lípidos, hidratos de carbono, ácidos orgánicos y etanol) sobre la actividad antimicrobiana de ciertos CAEs (carvacrol, eugenol, geraniol, timol y vainillina) en su forma libre. En esta primera parte, también se ha evaluado la influencia de estos componentes alimentarios sobre la actividad antimicrobiana de vainillina inmovilizada sobre partículas de óxido de silicio. Los resultados mostraron que la seroalbúmina bovina (BSA), el aceite de girasol y algunos carbohidratos, son los componentes alimentarios que mayormente inhibían total o parcialmente la actividad antimicrobiana de los CAEs libres evaluados. Sin embargo, se encontraron ciertas excepciones. En medios que contienen BSA la actividad antimicrobiana del geraniol no se ve inhibida. Lo mismo ocurría con el eugenol en medios que contienen aceite de girasol, o con carvacrol, eugenol, geraniol y timol en medios que contienen D-lactosa. Así mismo, en el estudio realizado sobre vainillina inmovilizada se confirmó el efecto inhibidor de la actividad antimicrobiana que ejercían las proteínas, lípidos y carbohidratos evaluados. A pesar de ello, se identificaron componentes (ácido cítrico y etanol) que mejoraban la actividad antimicrobiana, haciendo que el efecto bloqueante quedara superado. Estos resultados demuestran la importancia de considerar la composición de la matriz alimentaria a la hora de elegir un compuesto antimicrobiano.

Resumen

Tras el estudio del efecto de los distintos componentes presentes de forma natural en los alimentos, en el segundo capítulo se ha evaluado la aplicación de los CAEs frente al crecimiento y producción de guayacol por parte de un microorganismo alterante y termorresistente como es *Alyciclobacillus acidoterrestris* en zumo de naranja. Para ello, los CAEs inmovilizados sobre partículas de óxido de silicio fueron usadas con dos enfoques diferentes: como aditivos y como coadyuvantes. Los resultados de los ensayos han mostrado que la presencia de CAEs provoca una reducción microbiana y una inhibición de la producción de guayacol, la cual se mantiene tras la inmovilización de los antimicrobianos. Este hecho es de gran interés, ya que la inmovilización evita el problema de alteración organoléptica del producto, derivado de la aplicación de estos antimicrobianos en forma libre.

En el tercer capítulo se ha estudiado la actividad antiviral de los CAEs, tanto en forma libre como inmovilizados, contra el virus Tulane en agua. Los resultados han mostrado que, mientras que la aplicación de los CAEs en forma libre logró una reducción de la infectividad de apenas un 1 $\log_{10}$, concentraciones equivalentes de antimicrobiano inmovilizado redujeron la infectividad hasta alcanzar niveles no detectables (reducción superior a 4.5 $\log_{10}$). Por otro lado, se ha demostrado que el mecanismo antiviral está basado en la capacidad de los antimicrobianos inmovilizados de modificar o destruir la cápside viral, que es la responsable de otorgar al virus las propiedades infectivas. Además, se determinó que los CAEs inmovilizados no son citotóxicos a las concentraciones antivirales efectivas.

A pesar de la eficacia observada tras la inmovilización de los CAEs en partículas de óxido de silicio contra bacterias y virus, su aplicación práctica en la industria alimentaria presenta varios retos, siendo uno de ellos que los consumidores acepten esta tecnología. Por este motivo, en el último capítulo se ha estudiado la percepción que tienen los consumidores sobre el empleo de la nanotecnología en el procesado de alimentos. La valoración de los diferentes alimentos en los que la nanotecnología había sido aplicada en su procesado o envasado, en general fue positiva, mostrando la mayoría de los consumidores disposición a comprarlos. De todos los productos, los que presentaron una mejor valoración fueron aquellos en los que la nanotecnología no formaba parte del alimento. Considerando este

resultado, los CAEs inmovilizados que son aplicados como coadyuvantes alimentarios serían los mejores valorados, y, por tanto, podrían ser una excelente alternativa frente tratamientos convencionales de conservación, para controlar tanto virus y bacterias durante la producción y almacenamiento de los alimentos.

Resum

Els components dels olis essencials (COEs) han resultat ser efectius enfront d'una gran varietat de microorganismes. No obstant això, l'aplicació directa d'aquests components planteja reptes a causa de la seua baixa solubilitat i a l'alteració de les propietats organolèptiques dels aliments. En resposta a aquestes limitacions, la cerca de noves formes de dosificació d'aquests agents tan prometedors és vital per tal d'afavorir el seu ús en la indústria alimentària.

La present tesi doctoral està centrada en el desenvolupament i aplicació de sistemes antibacterians i antivirals basats en la immobilització covalent de COEs per a millorar la qualitat i seguretat dels aliments líquids.

En el primer capítol es va avaluar l'efecte de l'addició de components naturalment presents en els aliments (proteïnes, lípids, hidrats de carboni, àcids orgànics i etanol) sobre l'activitat antimicrobiana de certs COEs (carvacrol, eugenol, geraniol, timol i vanil·lina) en la seua forma lliure. En aquesta primera part, també s'ha avaluat la influència d'aquests components alimentaris sobre l'activitat antimicrobiana de vanil·lina immobilitzada sobre partícules d'òxid de silici. Els resultats van mostrar que la seroalbúmina bovina (BSA), l'oli de gira-sol i alguns carbohidrats, són els components alimentaris que majorment inhibien total o parcialment l'activitat antimicrobiana dels COEs lliures avaluats. No obstant això, es van trobar certes excepcions. En medis que contenen BSA l'activitat antimicrobiana del geraniol no es veu inhibida. El mateix ocorria amb el eugenol en medis que contenen oli de gira-sol, o amb carvacrol, eugenol, geraniol i timol en medis que contenen D-lactosa. Així mateix, en l'estudi realitzat sobre vanil·lina immobilitzada es va confirmar l'efecte inhibidor de l'activitat antimicrobiana que exercien les proteïnes, lípids i carbohidrats avaluats. Malgrat això, es van identificar components (àcid cítric i etanol) que milloraven l'activitat antimicrobiana, fent que l'efecte inhibidor quedara superat. Aquests resultats demostren la importància de considerar la composició de la matriu alimentària a l'hora de triar un compost antimicrobià.

Resum

Després de l'estudi de l'efecte dels diferents components presents de manera natural en els aliments, en el segon capítol s'ha avaluat l'aplicació dels COEs enfront del creixement i producció de guaiacol per part d'un microorganisme alterant i termo resistent com és *Alyciclobacillus acidoterrestris* en suc de taronja. Per a això, els COEs immobilitzats sobre partícules d'òxid de silici s'han usat amb dos enfocaments diferents: com a additius i com a coadjuvants. Els resultats dels estudis han mostrat que la presència de COEs provoca una reducció microbiana i una inhibició de la producció de guaiacol, la qual es manté després de la immobilització dels antimicrobians. Aquest fet és de gran interés, ja que la immobilització evita el problema d'alteració organolèptica del producte, derivat de l'aplicació d'aquests antimicrobians en forma lliure.

En el capítol tercer s'ha estudiat l'activitat antiviral dels COEs, tant en forma lliure com immobilitzats, contra el virus Tulane en aigua. Els resultats han mostrat que, mentre que l'aplicació dels COEs en forma lliure va aconseguir una reducció de la infectivitat de només 1 $\log_{10}$, concentracions equivalents d'antimicrobià immobilitzat van reduir la infectivitat fins a aconseguir nivells no detectables (reducció superior a 4.5 $\log_{10}$). D'altra banda, s'ha demostrat que el mecanisme antiviral està basat en la capacitat dels antimicrobians immobilitzats de modificar o destruir la càpsida viral, que és la responsable d'atorgar al virus les propietats infectives. A més, es va determinar que els COEs immobilitzats no són citotòxics a les concentracions antivirals efectives.

Malgrat l'eficàcia observada després de la immobilització dels COEs en partícules d'òxid de silici contra bacteris i virus, la seua aplicació pràctica en la indústria alimentària presenta diversos reptes. Un d'ells és que els consumidors accepten aquesta tecnologia. Per aquest motiu, en l'últim capítol s'ha estudiat la percepció que tenen els consumidors sobre l'ús de la nanotecnologia en el processament d'aliments. La valoració dels diferents aliments en els quals la nanotecnologia havia sigut aplicada en el seu processament o envasament, en general va ser positiva, mostrant la majoria dels consumidors disposició a comprar-los. De tots els productes, els que van presentar una millor valoració van ser aquells en els quals la nanotecnologia no formava part de l'aliment. Considerant aquest resultat, els COEs immobilitzats que són aplicats com a coadjuvants alimentaris

serien els millors valorats, i, per tant, podrien ser una excel·lent alternativa enfront dels tractaments convencionals de conservació, per a controlar tant virus com bacteris durant la producció i emmagatzemament dels aliments.

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1. PREAMBLE

1. PREAMBLE

The use of natural antimicrobials in food preservation processes is becoming increasingly widespread, driven not only by the imperative of food safety, but also by the search for improved product quality. This fact can be attributed mainly to three factors. First, there is a current trend among consumers to reject synthetic preservatives. In this context, consumer preference for minimal processing, freshness and superior quality products opens up the use of natural compounds in industrial scenarios. Secondly, traditional preservation methods, in particular heat treatments, are known to degrade nutrients and negatively affect the organoleptic properties of the product. Additionally, the reliance on energy-intensive sources like gas or fuel for these treatments contributes to greenhouse gas emissions and global warming.

In consideration of the aforementioned factors, the search for alternative treatments to conventional methods is necessary. Among them, treatment with purified plant-derived compounds has proven to be effective against a large number of microorganisms. However, their direct application significantly modifies the organoleptic characteristics of the products. In response to these limitations, new treatment methods with these promising agents need to be developed to enable their use in the food industry.

This PhD thesis has been developed in the framework of two different research projects: a) “Application of functionalized materials with antiviral, antibiofilm, antienzymatic and antimicrobial activity in the food industry (PID2021-128141OB-C21)” (2022-2025), funded by the National Research Plan of the Spanish Ministry of Economy and Competitiveness and b) “Non thermal pasteurization of liquid foods using green chemistry (TED2021-132035B-I00)” (2022-2024) funded by the Spanish Ministry of Economy and Competitiveness and European Union NextGenerationEU/PRTR, respectively. The main objective of the project "Application of functionalized materials with antiviral, antibiofilm, antienzymatic and antimicrobial activity in the food industry” was to evaluate the stability of bioactive substances of antimicrobial interest by immobilization in a support. These supports contain an inorganic material (amorphous silica particles) where the

different proposed biomolecules are anchored, allowing the compound to exert its action, and preventing its release into the product. On the other hand, one of the main objectives of the project "Non thermal pasteurization of liquid foods using green chemistry" was to evaluate the effect of food components naturally present in food (i.e. proteins, lipids, carbohydrates, organic acids, alcohols or minerals) on the antimicrobial activity of natural compounds.

Taking into account the above-mentioned objectives of the two projects, the present thesis assesses the possibility of using essential oil components as antiviral and antimicrobial agents to improve the quality and safety of liquid foods by using non-thermal methods.

In order to structurally show the specific outcomes obtained in this thesis, this document has been organized into five distinct sections:

- The Introduction reviews the state of the art of both the free and immobilized application of essential oil components (EOC) in fruit-derived products, serving as a case of study of liquid foods.
- The Objectives section outlines the primary and specific goals of this thesis.
- The Results section systematically presents the various results obtained. The information is structured into four chapters, which contain a reproduction of the scientific articles that are being peer-reviewed or are already published in different journals.
- After the presentation of the results in each chapter, they are analyzed collectively in the General Discussion section.
- Finally, the Conclusions and Future Perspectives section highlights the most important outcomes of the research and indicates possible future lines.

2. GENERAL INTRODUCTION [1]

[1] General introduction is based on the article: Gómez-Llorente, H., Fernández-Segovia, I., Pérez-Esteve, É., Ribes, S., Rivas, A., Ruiz-Rico, M., & Barat, J. M. (2023) Immobilization of natural antimicrobial compounds on food-grade supports as a new strategy to preserve fruit-derived foods. *Foods* (DOI: 10.3390/foods12102060).

1. Introduction

The consumption of natural healthy liquid products, such as milk, vegetable, or fruit derived foods (i.e., juices, soft drinks, jams, sauces, or wines), has increased in recent years owing to their convenience and acknowledged nutritional and functional properties. These properties are the result of their high content of antioxidants, calcium, fiber, polyphenols, and vitamins (A, C, and B group), and their low sodium and fat contents (Mani-López et al., 2019).

As liquid foods are perishable, their processing plays a crucial role in guaranteeing their safety and extending their shelf life. Liquid food preservation techniques are based mainly on the use of heat treatments or synthetic preservatives. Heat treatments (i.e., pasteurization and sterilization) enable a product's microbial load to be reduced or eliminated and allow the enzymes present in food to be deactivated and, thus, contribute to greater stabilization. Thermal pasteurization is considered the most appropriate methodology for some liquid foods. For example, in juices, U.S. FDA has stablished that to ensure the food safety, a 5-log reduction in the microorganisms that can cause spoilage (i.e., *Alicyclobacillus acidoterrestris*) and pose public health problems (i.e., *Escherichia coli* O157:H7 or *Salmonella enterica*) must be achieved (Food and Drug Administration, 2001). However, heat treatments cause loss of water-soluble and oxygen-labile nutrients, such as vitamins (Mieszczakowska-Fr et al., 2021), and undesirable organoleptic changes such as reduced fresh-like flavor (Corbo et al., 2009).

Another relevant factor to consider is the preservation methodology's carbon footprint. Heat treatments use gas or fuel as a source of energy and release greenhouse gas (GHG) emissions. Consequently, they contribute to global warming. As a piece of data, it can be stated that GHG emissions from juice pasteurization operations amount to 5.5 g CO_2/L of juice in a 96% heat recovery thermal system (Atuonwu et al., 2018). Considering that in most cases a juice is pasteurized at least twice (once immediately after extraction and again prior to bottling), the result is 11 g CO_2/L of juice. With these data, the carbon footprint that results from the pasteurization of liquid foods in Europe can be estimated at 1×10^5 tons CO_2/year.

On the other hand, the addition of synthetic preservatives, such as sorbates, benzoates, or sulfur dioxide, is very much questioned because their use can lead to dangerous health problems or antimicrobial resistance (McKernan et al., 2021), and such preservatives can be rejected by consumers for not being natural (Ribes et al., 2016).

In order to reduce the impact of thermal processing on the carbon footprint, and also on the sensory, nutritional, and functional properties of liquid foods, different cold pasteurization methods have been developed as an alternative to conventional preservation methodologies (Hu et al., 2023). These methods can be grouped into two groups: (i) physical methods, such as UV irradiation, high-pressure processing, high-intensity pulsed electric field, ultrasounds, filtration, etc., and (ii) chemical methods. Non-thermal physical treatments have been developed to preserve the sensory and nutritional properties of foods. However, they are ineffective against bacterial spores, are expensive, require complex equipment and management procedures, and can even have a bigger carbon footprint than conventional heat treatment (Atuonwu et al., 2018). In the application of unconventional chemical methodologies, it is important to highlight the use of naturally occurring antimicrobial compounds to ensure food safety and quality due to their broad-spectrum antimicrobial activities and biocompatibility (Quinto et al., 2019).

Having this in mind, the different applications of naturally occurring antimicrobial compounds as an alternative to conventional preservation techniques used in liquid foods were reviewed, focusing on fruit-derived foods as a case of study. For this purpose, a systematic review was carried out on the direct application of natural antimicrobials (free form) to fruit-derived products, and their use after covalent immobilization on the surface of different food-grade supports, to be employed as food preservatives or food processing aids to avoid modifying the sensory properties of the food. This review includes the advantages and disadvantages of using natural antimicrobials according to their formulation (free or immobilized forms) and their application form on the food product (preservative or processing aid). A description of the main methodologies for the covalent immobilization of natural antimicrobials and the most relevant characterization

techniques to verify covalent grafting and bioactivity is also included. Finally, future perspectives of these alternative preservation technologies are proposed.

2. Methodology

A systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Page et al., 2021) was conducted to compile the most relevant studies using natural antimicrobials in fruit-derived foods using two dosage forms: free (Section 3) or immobilized (Section 4). The selection process was carried out in different stages, as described below.

2.1 Identification stage

The selection process proposed by Bhardwaj et al. (2023) and Rifat et al. (2022) was followed for this stage. Three reviewers independently carried out the literature searches and checked the number of records. The employed research databases were Scopus, Web of Science, and PubMed. The search strategy was carried out by taking into account that "naturally-occurring antimicrobial compounds" covered the following terms: essential oils, fatty acids, lysozyme, chitosan, bacteriocin, bacteriophage. Subsequently, the following combined queries ("naturally-occurring antimicrobial compounds" AND "wine") OR ("naturally-occurring antimicrobial compounds" AND "juice") OR "naturally-occurring antimicrobial compounds" AND "jam") OR ("naturally-occurring antimicrobial compounds" AND "beverage") were used. Of all the found reports, all those whose publication range was between 2017 and 2023, and written in English, were selected.

2.2 Screening stage

The search results were exported to Mendeley reference manager and duplicates were removed.

2.3. Eligibility Stage: Inclusion and Exclusion Criteria

Documents were screened according to the inclusion and exclusion criteria in the title and abstract. Eligible articles were evaluated by three reviewers after considering the inclusion and exclusion criteria for the full text (**Table 1**).

Table 1. Inclusion and exclusion criteria for Section 3 and Section 4.

Section	Item	Inclusion criteria	Exclusion criteria
		Eligibility stage	
3 and 4	Type of reports	Scientific articles	Reviews, books, book chapters, conference proceedings, or theses.
3 and 4	Title, abstract, and keywords	Availability of title, abstract, and keywords; AND description and evaluation of the antimicrobial activity of naturally occurring antimicrobial compounds in fruit-derived products.	Evaluation of the antimicrobial activity of encapsulated natural antimicrobial compounds; OR in vitro studies not applied to food derived from fruit or for biomedical applications OR when their use is as a film, emulsion, or coating.
3	Title, abstract, and keywords	Application of naturally occurring compounds in the free form.	Evaluation of antimicrobial activity in the immobilized dosage form.
4	Title and abstract	Application of naturally occurring compounds in the immobilized form; AND evaluation of the antimicrobial activity of naturally occurring compounds as food preservatives or processing aids.	Evaluation of antimicrobial activity in the free form or for biomedical applications.
		Included stage	
3 and 4	Full text	Full-text availability; AND proper description of materials, methods, and results; AND description and evaluation of the antimicrobial activity of naturally occurring compounds in fruit-derived products.	No full text is available; OR the materials and methods are not described or are incomplete; OR the application is in vitro or applied to food that is not derived from fruit.
3	Full text	Description and evaluation of the antimicrobial activity of naturally occurring compounds in the free dosage form.	Use of immobilized naturally occurring antimicrobial compounds.
4	Full text	Description and evaluation of the antimicrobial activity of naturally occurring compounds in the immobilization form.	Use of naturally occurring antimicrobial compounds in the free form; OR immobilized antimicrobial compounds with biomedical purposes.

Variations in the number or records in each stage were checked and any discrepancies were assessed by the reviewers to make a final decision.

Information about the number of records included in this review and the summary of the selection process of the included articles are shown in **Figure 1**.

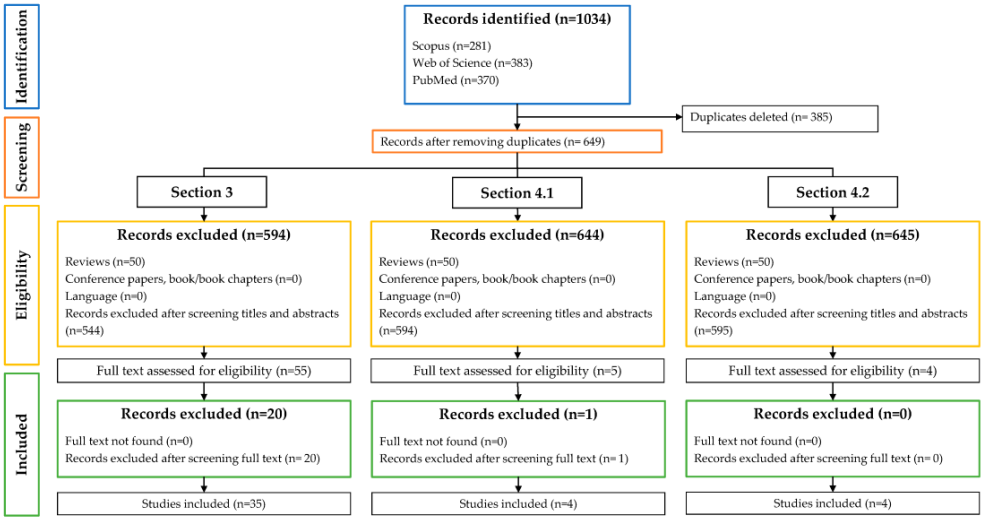

Figure 1. Summary of the selection process of the included articles.

3. Using Natural Antimicrobials in Fruit-Derived Foods

The systematic review on the use of natural antimicrobials in fruit-derived foods revealed that essential oil components (EOCs), bacteriocins, polysaccharides, organic acids, or bacteriophages are the most frequent molecules employed as preservatives in fruit-derived foods, probably due to their consideration as GRAS (generally recognized as safe) products by the U.S. FDA (Carvalho et al., 2023).

EOCs are a mixture of different compounds, such as terpenes, alcohols, phenols, etc., generated from plants. Free hydroxyl functional groups (-OH) are mostly responsible for their antimicrobial activity (Thomas-Popo et al., 2019). Bacteriocins are the peptides obtained from bacteria that are capable of changing the permeability of microorganisms by provoking their lysis. Of all the bacteriocins,

nisin, iturin A, natamycin, bovicin, and thurincin H are proposed as antimicrobials (Karaman et al., 2019; Liao et al., 2017; Ribeiro et al., 2022; Ruiz-De Anda et al., 2022 and Shi et al., 2018). The most important polysaccharide is chitosan, a biopolymer generated by the deacetylation processes of chitin. Its antimicrobial action is based mainly on the interaction between chitosan cationic groups and microorganisms (Miot-Sertier et al., 2022). Organic acids cover different compounds that present a deprotonated carboxyl group at a neutral pH. The interaction between the active group and a microorganism's membrane is the main antimicrobial mechanism (Wang et al., 2017). Bacteriophages are viruses that penetrate a specific bacterial host, spread within the host, and release more phages after cell lysis (Guo et al., 2021).

The antimicrobial activity of the naturally occurring compounds was tested in different fruit-derived products (i.e., wine, fruit juices, or soft drinks) against bacteria, such as Escherichia coli, Salmonella enterica, and *Listeria monocytogenes*, yeasts, such as *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, and *Saccharomyces cerevisiae*. In the reviewed studies, the authors reported remarkable efficacy for the microbiological control of all the fruit-derived foods. Table 2 summarizes the different studies that evaluated the antimicrobial efficacy of the aforementioned compounds in fruit-derived foods.

Despite the demonstrated in vivo inhibitory efficacy of antimicrobials, some studies have revealed that their direct addition to food presents certain limitations that stem from their intrinsic physico-chemical properties. Mitropoulou et al. (2020) and Thomas-Popo et al. (2019) expressed that essential oils (EOs) and their components (EOCs) are poorly soluble in aqueous media and highly volatile when studied for antimicrobial purposes in fruit juices. Campion et al. (2017) indicated that nisin is more stable at an acid pH when tested in milk and apple juice media. Liao et al. (2017) reported that the temperature and salt concentration of media can affect nisin activity for apple juice stabilization.

Table 2. Relevant studies by applying free natural antimicrobial compounds in fruit-derived foods

Antimicrobial	Food matrix	Target microorganisms	Effect on food product	Reference
Essential oils or their components				
Combination of carvacrol and nisin	Apple juice	*Escherichia. coli O157:H7*	The nisin and carvacrol combination caused the complete inhibition of the bacterium after 3 h of incubation at room temperature.	Campion et al., 2017
Oregano and thyme EOs	Wine	*E. coli, Salmonella enterica*	Both EOs were more active against *S. enterica* than *E. coli* when added to wine.	Friedman et al., 2017
Vanillin and cinnamaldehyde	Coconut water	*Salmonella enterica* serovar *Typhimurium*	The complete elimination of the bacterium was achieved when adding 100 µg/mL of cinnamaldehyde, while the ddition of vanillin only delayed microorganism growth. Both antimicrobials produced undesirable sensory characteristics at 100 µg/mL.	Beristaín-Bauza et al., 2018
Thymol	Calamansi Juice	*E. coli 0157:H7, S. enterica* serovar *Typhimurium*	The use of thymol (20 mM) led to the total elimination of all the tested microorganisms. Nevertheless, thymol incorporation negatively affected the sensory profile of the food-derived product.	Chung et al., 2018
Cinnamon leaf EO	Orange juice	*Saccharomyces cerevisiae*	Cinnamon EO (650 mg/mL) brought about a reduction in yeast of *ca*. 4 log CFU/mL.	Sánchez-Rubio et al., 2018
Thymbra capitata EO	Pomegranate juice	Aerobic mesophilic bacteria, *Streptococcus thermophilus,* yeasts and molds	For all the microorganisms, the addition of the EO at 0.125 % (v/v) provoked a significant reduction (4 log CFU/mL) when incubated at 4 and 25 °C, while no impact on physico-chemical characteristics was detected.	Charfi et al., 2019
Mint EO, carvacrol and natamycin	Apple juice	*Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii*	The employed antimicrobial preservatives were able to reduce ethanol formation thanks to microorganisms inhibition.	Karaman and Sagdic, 2019
Isoeugenol	Pineapple juice	*E. coli O157:H7, S. enterica,* and *Listeria monocytogenes*	The addition of 0.5 µL/mL of the EOC managed to reduce > 5 log CFU/mL in all the tested microorganisms. The antimicrobial modified some sensory parameters.	Thomas-Popo et al., 2019
Eucalyptus globulus EO (EGEO)	Orangina fruit juice	*S. cerevisiae*	EGEO in combination with heat treatment (70 °C for 2 min) at concentrations ranging from 0.8 to 4 µL/mL was effective in reducing *S. cerevisiae* growth in orangina juice.	Boukhatem et al., 2020

Table 2. Continuation

Antimicrobial	Food matrix	Target microorganisms	Effect on food product	Reference
Essential oils or their components				
Citrus medica and *Cinnamomum zeylanicum* EOs	Wine	*Oenococcus oeni*, *Pediococcus pentosaceus*, *Gluconobacter cerinus*, *Brettanomyces bruxellensis*, *Candida zemplinina*, *Hanseniaspora uvarum*, *Pichia guilliermondii* or *Z. bailii*	Both extracts allowed shelf life to extend from 9 days (when not applying EOs) to 18 and 74 days when using *C. medica* and *C. zeylanicum* EOs, respectively. The addition of EOs modified the sensory profile. The product was rejected when EOs concentration was raised to up to 0.010 %.	Mitropoulou et al., 2020
Citrus lemon and *Citrus reticulata* EOs	Orange juice	*Lactobacillus brevis, L. mesenteroides*	The use of both EOs alone (0.5 µL/mL) or combined caused bacterial reduction.	Pedrosa et al., 2020
Carvacrol, thymol and trans-cinnamaldehyde	Apple juice	*Z. rouxii*	All the EOCs provoked fungi reduction of *ca*. 100% when applied in apple juice.	Wang and Sun, 2020
Vanillin	Apple juice	*S. enterica* serovar Typhimurium	The microorganism was inactivated after 75 min at 45 ºC using 3.2 mg/mL of vanillin.	Bai et al., 2021
Cinnamon bark and thyme EOs, and thymol	Tomato juice	*L.monocytogenes*	The combined use of EOCs at 0.6250 µL/mL showed the best antimicrobial results. The bacterium was totally eliminated (>5.2 log CFU/mL) after 24 h of incubation at both 25 and 10 °C in tomato juice.	Kim et al., 2021
Pistacia lentiscus and *Fortunella margarita* EOs	Fruit juice (lemon, apple, blackcurrant)	*Aspergillus niger, S. cerevisiae*	The combination of EOs (*P. lentiscus* EO 0.2% (w/w) and *F. margarita* EO 0.006% (w/w) reduced fungi growth to < 100 spores/mL, while treatment was not effective against *S. cerevisiae*. Higher concentrations of EOs than those tested were rejected because they produced an intense bitter taste.	Mitropoulou et al., 2022
Thymol and trans cinnamaldehyde	Apple juice	*Z. rouxii*	The use of 0.125 and 1.25 of thymol and *trans* cinnamaldehyde mg/mL totally reduced in apple juice	Wang et al., 2022
Melissa officinalis EO (MEEO)	Watermelon juice	*L. monocytogenes*	The use of 2 µL/mL of MEEO resulted in complete bacterial growth reduction from day 2 to day 7 in inoculated watermelon juice.	Carvalho et al., 2023

Table 2. Continuation

Antimicrobial	Food matrix	Target microorganisms	Effect on food product	Reference
			Bacteriocins	
Nisin	Apple juice	Aerobic bacteria, molds and yeasts	The addition of nisin lowered the aerobic bacteria counts, while no changes appeared for molds and yeasts. The treatment with the antimicrobial did not change the product's color.	Liao et al., 2018
Iturin A	Orange juice	*S. cerevisiae*	The addition of iturin A (0.76 mg/mL) completely inhibited the target microorganism after 4 days of incubation.	Shi et al., 2018
Lactobacillus plantarum Cys5-4	Orange juice	*E. coli, S. enterica*	The application of the bacterium (128 AU/mL) reduced the *E. coli* population *ca*. 3 log CFU/mL after 5 days of incubation, while no antimicrobial effects were shown for *S. enterica*.	Tenea and Barrigas, 2018
Nisin	Coconut water	Yeasts, molds and total coliforms	The use of nisin (50 ppm) reduced the counts of both yeasts and molds (below 1 log CFU/mL), while the total coliforms were not detected. The sensory profile did not change after adding nisin.	Sumonsiri ,2019
Nisin	Apple juice	*E. coli, Listeria innocua*	Treatment with nisin (500 IU/mL) reduced 1.5 and 3 log CFU/mL for *E. coli* and *L. innocua,* respectively, after 30 min of incubation at 37 °C. The pH of the product was lower *(p<0.05.)* because nisin is stabler at an acid pH.	Mok et al., 2020
Nisin	Orange Juice	Total aerobic bacteria	The combination of thermosonication and nisin treatment led to remarkable bacterial reduction. The use of nisin increased the product's sensory acceptability.	Zhao et al., 2021
Kenaf seed peptides	Mango juice, pineapple juice	*S. enterica* serovar Typhimurium*, E. coli, L.monocytogenes*	The antimicrobial compound (3000 mg/mL) reduced all the tested bacteria (> 5 log CFU/mL) for 150 days in both media, while no color changes were detected.	Arulrajah et al., 2022
Bovicin HC5 and nisin	Pineapple, orange, papaya, grape, mango, and apple juices	*Alicyclobacillus acidoterrestris*	Treatment with 80 AU/mL of bovicin or nisin totally eliminated the bacterium vegetative cells and the thermal resistance of their endospores.	Ribeiro et al., 2022
Thurincin H	Orange juice	*E. coli, L. innocua*	40 µg/mL of the antimicrobial compound reduced *L. innocua* by 5.5 log CFU/mL, but only 1 log CFU/mL of *E. coli.*	Ruiz-De Anda et al., 2022

Table 2. Continuation

Antimicrobial	Food matrix	Target microorganisms	Effect on food product	Reference
		Bacteriocins		
Lactobacillus acidophilus NX2-6	Apple juice	*A. acidoterrestris*	The bacterium growth was inhibited by adding 0.2 % of supernatant containing acidocin NX2-6 at 28 °C. The addition of the antimicrobial increased the quality the product. Concretely, it was enlarged the storage time in a transparent and precipitation-free state.	Sun et al., 2022
Pediococcus acidilactici NCDC 252	Apple juice, apricot pulp and pre-pasteurized wine	*E. coli*	The use of 1 mg/mL of the antimicrobial reduced the *E. coli* population by *ca.* 3 log CFU/mL.	Dhanda et al., 2023
		Polysaccharides		
Chitosan	Wine	*Acetobacter malorum* and *Acetobacter pasteurianus*	The use of chitosan totally inhibited *A. pasteurianus,* while the same treatment reduced the *A. malorum* population by 50 % after 15 days of inoculation.	Valera et al., 2017
Chitosan	Wine	*Brettanomyces bruxellensis*	Antimicrobial activity depended on the strain of the used microorganism. Indeed 41 % of the assayed strain were reduced by chitosan.	Paulin et al., 2020
Chitosan	Wine	*S. cerevisiae,* acetic acid bacteria strains, lactic acid bacteria strains and *O. oeni*	The use of chitosan was effective for lactic acid bacteria and *O. oeni,* but acetic acid bacteria and *S. cerevisiae* barely reduced.	Miot-Sertier et al., 2022
		Organic acids		
Gallic acid and feluric acid	Apple juice	*E. coli, L. innocua*	The application of gallic acid (10 mM) and ferulic acid (1 mM) was able to reduce both microorganisms from 6 log CFU/mL to below the detection limit.	de Oliveira et al., 2019
β-resorcylic acid and caprylic acid	Orange juice	*S. enterica* serovar Typhimurium	The combination of both antimicrobial compounds (8.43 mM of β-resorcylic acid and 0.10 mM of caprylic acid) lowered the temperature needed to reach the microbial parameters corresponding to pasteurization. Color and flavor did not change after treatment.	Kim and Rhee, 2020
p-Coumaric acid	Apple juice	*A. acidoterrestris*	The addition of the antimicrobial compound (0.4 mg/mL) accelerated the degradation of vegetative cells from 5 to 3 days at 4 °C. The study did not show any changes in the aroma profile after treatment.	Li et al., 2022

Table 2. Continuation

Antimicrobial	Food matrix	Target microorganisms	Effect on food product	Reference
Bacteriophages				
Lytic phage vB_SalS-LPSTLL	Apple juice	*S. enterica* serovar Typhimurium	The addition of the bacteriophage reduced ($p<0.05$) bacteria counts by up to 0.5 log CFU/mL in apple juice. Treatment did not change the sensory quality of the fruit-derived product.	Guo et al., 2021

In addition to all these limitations, some reports have shown that the incorporation of these compounds modifies some food sensory attributes. The study of Beristaín-Bauza et al. (2018) evaluated the sensory impact of 0–100 µg/mL of cinnamaldehyde and vanillin in coconut water. These authors reported the lowest general acceptability values at the highest concentration for both antimicrobial compounds. Chung et al. (2018) tested the addition of thymol to different citrus extracts (lime, lemon, and calamarsi) and quantified a lower overall acceptability ($p < 0.05$) by incorporating thymol at concentrations up to 2 mM. Further research rated the appearance, odor, taste, aftertaste, viscosity, and overall acceptance parameters at different concentrations of isoeugenol (0–1 µL/mL) added to pineapple juice (Thomas-Popo et al., 2019). The results showed a significant decrease ($p < 0.05$) in the odor, taste, aftertaste, and overall acceptance scores, while no changes were recorded for the appearance and viscosity characteristics. Using EOs from Citrus medica and Cinnamomum zeylanicum for microbiological wine stabilization (Mitropoulou et al., 2020), aroma and taste assays were carried out. The results indicated that the incorporation of EOs significantly affected both parameters. Indeed, the product was rejected when concentrations over 0.010 % of EOs were added because EOs masked the wine taste and also formed an oily layer on wine.

The interaction of antimicrobial compounds with food matrix components also limits their application in food products (Charfi et al., 2019; Dhanda et al., 2023; Guo et al.,2021; Mitropoulou et al., 2022; Ruiz-De Anda et al., 2022; Tenea et al., 2018 and Thomas-Popo et al., 2019). Certain nutrients in food can have a protective effect on microorganisms; therefore, it would be necessary to use higher concentrations of natural antimicrobials. However, increasing the antimicrobial effective dose can result in more limitations that derive from applying a high concentration of certain natural antimicrobial compounds, as previously highlighted.

For all these reasons, research is currently exploring new alternative dosage forms of natural antimicrobials to be used as food preservatives or processing aids, such as encapsulation of immobilization. The first alternative consists of trapping an active agent (i.e., a natural antimicrobial) in a carrier material to enhance its later

release in the food or in the gastrointestinal tract. Immobilization, in contrast, consists of anchoring the active biomolecule on the surface of the support. This technology not only makes it possible to preserve the native antimicrobial properties of the active biomolecule but also prevents its leaching into the food matrix due to the creation of covalent bonds between the support and the antimicrobial compound.

Having this in mind, covalent immobilization is presented in this work as an eco-friendly postharvest technology with great possibilities for application in the preservation of fruit-derived foods.

4. Using Immobilized Antimicrobials in Fruit-Derived Foods

The use of immobilized natural antimicrobials to preserve or extend the shelf life of fruit-derived products has increased in recent years owing to their marked antimicrobial effectiveness and good capacity to cushion their sensory and stability impact on foods after grafting (Peña-Gómez et al., 2019; Ribes et al., 2017 and Ruiz-Rico et al., 2017).

Immobilization refers to the chemical, physico-chemical, or electrostatic binding of bioactive molecules to a surface. Chemical immobilization involves the formation of at least one covalent bond between the surface and the target biomolecule, which represents the most permanent and irreversible form of coupling. Covalent linkage involves strongly bounding the compound of interest with a potentially longer shelf life, greater bioactivity, and lower toxicological risk (Silva et al., 2016). The immobilization of natural antimicrobials on the surface of different materials is an approach that allows contact-killing materials to be obtained through antimicrobial molecules that are covalently attached to the surface. With this immobilization procedure, antimicrobials are exposed to the external environment, which enables direct contact between the immobilized molecule and the target microorganism (Rosner et al., 2021).

According to this systematic review, these antimicrobial systems can be applied to fruit-derived foods in different processing stages: (i) as a food additive (preservative) present in the final product and (ii) as food processing aids that are

absent in the final product (see Figure 2). This section focuses on discussing application cases of immobilized natural antimicrobials in fruit-derived products by differentiating these two application approaches.

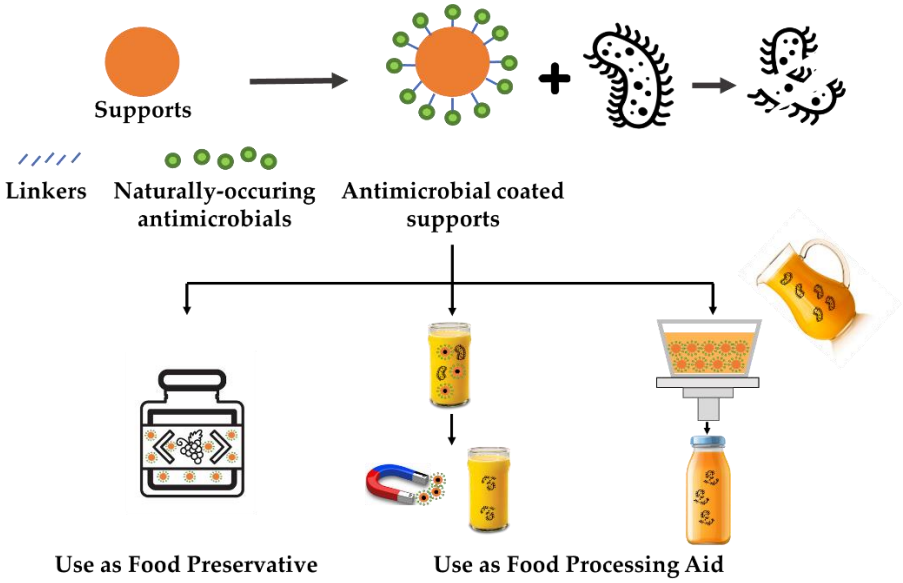

Figure 2. Schematic representation of the main uses of natural antimicrobial compounds immobilized on food-grade supports for fruit-derived food preservation.

4.1 Use as Food Preservatives

In the last few years, the design of immobilized antimicrobial systems as food preservatives to control or prevent microbial spoilage in fruit-derived foods has grown. Table 3 summarizes the four studies found in the systematic review about immobilized natural antimicrobial compounds on food-grade supports to be used as food preservatives in fruit-derived products. For all the identified applications, besides the description of the support, immobilization technique, target microorganism, and the food matrix that is the study object, the physico-chemical and sensory impact after their incorporation into the matrix is discussed.

In this context, Ribes et al. (2017) conducted the first work, in which EOCs immobilized by an imine bond on the surface of silica supports were employed as

promising antifungal agents to control strawberry jam decay without altering the final product's sensory perception. Based on the marked antimicrobial activity of these promising preservatives, Ribes et al. (2019) also investigated the synergistic effect of EOCs immobilized on the surface of silica particles against the bacteria and yeasts present in fruit juice and their influence on the food matrix. This work demonstrated the feasibility of combining immobilized antimicrobials to improve the microbial stabilization of fruit juice given that immobilization masks the undesirable aroma of EOCs in the food matrix according to both the gas chromatography and sensory evaluation results. Similarly, the antimicrobial activity of thymol immobilized on hollow mesoporous silica particles (HMSNs) in a real food system was investigated for the first time by Liu et al. (2022). It is noteworthy in this study that the antimicrobial agent was covalently grafted to the silica support by running a reaction with 3-(triethoxysilyl)-propyl isocyanate, which resulted in carbamate bonding instead of the imine bonding described in the above-mentioned examples. The EOC immobilized on HMSNs showed an excellent potential for enhancing the antimicrobial activity of thymol against foodborne bacteria. It also reduced the impact of EOCs on the final product's physico-chemical properties, such as color, pH, and soluble solids content. However, the impacts on the most relevant organoleptic properties (aroma and flavor) were not evaluated. Recently, different antimicrobial systems based on the covalent immobilization of chitosan for the microbial control of apple juice were developed by Ruiz-Rico et al. (2023). The use of chitosan-coated supports as food preservatives in juice reduced the food matrix's microbial load, which increased its shelf life, although the impact on juice was not evaluated. All these examples confirm the potential of antimicrobial systems based on immobilized natural antimicrobials as food preservatives in the food industry after regulatory authorities have approved them.

Finally, it is worth mentioning that all these reviewed systems present common characteristics: (i) the bioactive compound is covalently immobilized on the surface of the support; (ii) immobilized bioactive compounds exhibit greater antimicrobial or antifungal activity than their free form; (iii) the use of these novel preservatives does not modify the final product's physico-chemical or sensory properties.

Table 3. Relevant studies that applied natural antimicrobials covalently immobilized on the surface of different supports as antimicrobial and antifungal systems in fruit-derived foods.

Antimicrobial	Support	Immobilization technique	Target microorganisms	Food matrix	Effect on food product	Reference
Eugenol and thymol	MCM-41 microparticles	(1) Silanization of support with APTES. (2) Covalent imine bonding between the amino group of APTES and the aldehyde group of the previously modified EOC by Schiff reaction.	*Aspergillus flavus*, *A. niger*, *Penicillium expansum*, *Z. bailii* and *Z. rouxii*	Strawberry jam	Jams prepared with immobilized eugenol exhibited no mold and yeast growth. Immobilization reduced the intensity of eugenol and thymol aromas > 92% and 96%, respectively.	Ribes et al. (2017)
Eugenol, carvacrol and vanillin	MCM-41 microparticles	(1) Silanization of support with APTES. (2) Covalent imine bonding between the amino group of APTES and the aldehyde group of previously modified EOC by Schiff reaction.	*E. coli* and *Z. rouxii*	UHT-processed apple and grape juices	In apple juice, 0.05 mg/mL and 0.25 mg/mL of immobilized eugenol and vanillin, respectively, caused *E. coli* inhibition. *Z. rouxii* growth was inhibited with concentrations > 0.05 mg/mL and 0.125 mg/mL of immobilized eugenol and carvacrol, respectively. In grape juice, immobilized eugenol and 0.125 mg/mL of immobilized vanillin inhibited *E. coli* growth. Immobilization masked the characteristic undesirable aroma of the tested EOCs.	Ribes et al. (2019)

Table 3. Continuation.

Antimicrobial	Support	Immobilization technique	Target microorganisms	Food matrix	Effect on food product	Reference
Thymol	Hollow mesoporous silica nanoparticles, MCM-41 and amorphous silica particles.	Direct thymol immobilization by the co-condensation synthesis approach to obtain silica supports. Previous reaction of thymol with 3-(triethoxysilyl)-propyl isocyanate (TEPIC) to yield the corresponding alkoxysilane derivative by carbamate bonding to be used in the synthesis of the functionalized supports.	*E. coli*	Commercial apple juice	Immobilized thymol (0.2 mg/mL) inhibited *E. coli* growth in apple juice. Physico-chemical properties of apple juice were hardly influenced by the functionalized silica particles.	Liu et al. (2022)
Chitosan	MCM-41 microparticles and amorphous silica microparticles.	(1) Silanization of supports with TEPIC. (2) Covalent urea bonding between isocyanate groups of TEPIC and amino groups of chitosan.	*Z. bailii*	Commercial pasteurized apple juice	The addition of antimicrobial supports to apple juice totally inhibited *Z. bailii* development after 15 days of refrigerated storage (< 0.5 log CFU/mL).	Ruiz-Rico et al. (2023)

4.2. Use as Food Processing Aid

The second approach of using immobilized natural antimicrobials in fruit-derived foods does not imply the permanence of particles in food to exert their antimicrobial effect. So in this case, instead of considering the antimicrobial supports to be food preservatives, they should be taken as processing aids.

In the systematic review of this application, four works used immobilized antimicrobials as processing aids. Within this framework, Song et al. (2019) employed iron oxide nanoparticle–polydopamine–nisin composites with magnetic characteristics to control *A. acidoterrestris* growth in apple juice and recovered particles after treatment. After demonstrating that the juices treated with functionalized magnetic particles were not influenced in physico-chemical and sensory terms, as well as the non-toxicity and biosecurity of composites, the authors highlighted this innovative antimicrobial system as a promising tool to control *A. acidoterrestris* contamination in the juice industry.

Conversely, the other three studies applied the immobilization of natural antimicrobial compounds on food-grade supports as a strategy to create filtration systems for the cold pasteurization of liquid fruit-derived foods. The main objective of these filtration systems was to remove spoilage or pathogenic microorganisms from liquid fruit-derived products (juices or wine) through the retention and/or the disruption of the bacterial cell wall after the interaction with the antimicrobial compound (Ruiz-Rico and Barat, 2021). Zhang et al. (2021) reported employing nisin-coated polyvinylidene difluoride microfiltration membranes (pore diameter of 0.22 µm) to eliminate *A. acidoterrestris* contamination from apple juice due to the antibacterial action of nisin and the retention of spores on the membrane surface. In a different approach, Peña-Gomez et al. (2019) developed novel filtering materials based on silica microparticles (50 µm) functionalized with EOCs as an alternative cold pasteurization method for apple juice by depth filtration. In a first assay, the developed filtration system was able to reduce the *E. coli* load inoculated in pasteurized apple juice of at least 5 logarithmic reduction values (LRVs). In addition, employing antimicrobial particles for the filtration of fresh juices was able to microbiologically stabilize the non-thermally treated apple juice, which resulted

in juice with high microbial stability and quality. This suggests that this filtration technology is a promising alternative to existing pasteurization technologies that apply heat. In another work, Ruiz-Rico et al. (2021) evaluated filter aids based on the covalent immobilization of different EOCs and other phenolic compounds on wine microbiological stabilization. These filtering aids presented some advantages over standard filtration materials, such as minimal impact on wine sensory characteristics and high removal capacity. Likewise, they could be used for clarification, microbiological stabilization, and sterile filtration in a single continuous treatment, thus reducing wine losses and energy costs by compiling different traditional filtration stages in a single step, as well as enhancing the treatment's overall hygiene and security. However, the authors pointed out the need to improve the stability of grafting and the reuse conditions or filter life before being applied in the food industry. More information about the specific natural antimicrobials, supports, immobilization techniques, and target microorganisms studied in the described examples can be found in Table 4.

These described filtration systems differ from micro- and ultrafiltration techniques, which have been extensively studied and implemented for the cold pasteurization of drinks on an industrial scale, in terms of removal capacity and their impact on the properties of filtered drinks. The mechanism of action of micro- and ultrafiltration (pore size from 0.001 µm to <0.1 µm for ultrafiltration, and from 0.1 µm to 10 µm for microfiltration) is the physical retention of all the molecules and organisms bigger than the pore size. In contrast, filtration systems based on membranes or particles functionalized with natural antimicrobials exhibit a larger pore size or filtration channels that only allow the partial retention of microorganisms and food matrix components. Therefore, they act by the combined effect of cell retention and cell damage due to the specific interaction with the antimicrobial compound by preserving the nutritional, functional, and sensory properties of the filtered drink.

Table 4. Relevant studies that applied natural antimicrobials covalently immobilized on the surface of different supports as antimicrobial processing aids in fruit-derived foods.

Antimicrobial	Support	Immobilization technique	Target microorganisms	Food matrix	Effect on food product	Reference
Nisin	Iron oxide nanoparticles	(1) Coating the particle's surface with dopamine. (2) Covalent attachment of nisin to the polydopamine-coated support.	*A. acidoterrestris*	Apple juice	The samples treated with 2.5 mg/mL of nisin immobilized on iron oxide nanoparticles for 30, 60, and 120 min inhibited *A. acidoterrestris* growth. The physico-chemical and sensory properties of apple juices were not influenced by nisin-coated nanoparticles. Supports were magnetically recovered from the food matrix.	Song et al. (2019)
Eugenol and vanillin	Amorphous silica microparticles	(1) Silanization of support with APTES. (2) Covalent amine bonding between the amino group of APTES and the aldehyde group of pure vanillin or previously modified eugenol by Schiff reaction.	*E. coli* and native flora	Apple juice	The use of coated silica microparticles in depth filtration resulted in 4–5 LRVs of *E. coli* in juice. The eugenol-functionalized particles proved a more adequate support for removing the product's microbial load without affecting the physico-chemical parameters or sensory profile of juice.	Peña-Gomez et al. (2019)

Table 4. Continuation.

Antimicrobial	Support	Immobilization technique	Target microorganisms	Food matrix	Effect on food product	Reference
Nisin	Polyvinylidene difluoride microfiltration membrane	(1) Coating the membrane surface with dopamine. (2) Covalent attachment of nisin to the polydopamine-coated membrane by Michael addition and Schiff base and Michael reactions.	*A. acidoterrestris*	Apple juice	The use of a coated membrane grafted with nisin resulted in 5.8 LRVs of *A. acidoterrestris* in juice. The modified membrane exhibited reliable stability in different membrane cleaning procedures.	Zhang et al. (2021)
Eugenol, vanillin and trans-ferulic acid	Amorphous silica microparticles	(1) Silanization of support with APTES. (2) For eugenol and vanillin, covalent amine bonding between the amino group of APTES and the aldehyde group of pure vanillin or previously modified eugenol. For ferulic acid, covalent amide bonding between the amino group of APTES and the carboxylic acid of ferulic acid	*Acetobacter aceti*, *Lactobacillus plantarum*, *B. bruxellensis*, *Z. bailii* and *S. cerevisiae*	White wine	PHE-functionalized filters were capable of reducing 3 LRVs, and eugenol was the most effective compound. The eugenol-functionalized supports had a very low impact on the physico-chemical parameters.	Ruiz-Rico et al. (2021)

5. Immobilization of Natural Antimicrobials on Food-Grade Surfaces

After identifying and analyzing the different examples of applying immobilized natural antimicrobials, this section describes the immobilization approaches in detail to provide a guideline of the synthesis and characterization of these antimicrobial systems for future developments.

Chemical immobilization can be a complex process for preserving the antimicrobial properties of the target molecule on a specific surface. The specific properties of the substrate surface, such as composition, charge, hydrophilicity/hydrophobicity, chemical stability, roughness, and geometry, as well as antimicrobial characteristics, including molecular structure, charge, and molecular size, should be considered for the bonding process. In addition, covalent immobilization can alter the conformational molecule structure by altering its mechanism of action. Therefore, coupling strategies should be carefully evaluated to optimize the attachment and maintenance of antimicrobial properties (Silva et al., 2016).

5.1. Substrate Surfaces for the Immobilization of Natural Antimicrobials

The substrate surfaces for the immobilization of biomolecules are diverse in terms of their features and properties, but the main characteristic they must present for coupling is the presence of sufficient functional groups for attaching the target molecule (Zucca and Sanjust, 2014). Otherwise, it may be necessary to modify its surface for immobilization (Goddard et al., 2007). Of the food-grade materials permitted to come into contact with food, this review identified different organic and inorganic substrates. Organic materials, such as cellulose, chitosan, or synthetic polymers (i.e., polystyrene, polyethylene, polyamide, or polyvinylidene difluoride), are commonly used substrates for biomolecule immobilization. The chitosan structure presents many hydroxyl and amine groups that enable the effective grafting of biomolecules without involving any modification (Zdarta et al., 2018). Other organic substrates present low or non-reactive functional moieties that require previous surface activation for specific grafting reactions (Kong and Hu, 2012). Of the available inorganic substrates, ceramic materials such as silica, clay,

sand, or glass, and metallic materials such as iron oxide, zinc oxide, titanium dioxide, or stainless steel are suitable support materials for biomolecule immobilization. Silica is the most widely used inorganic support material for biomolecule immobilization, which can be obtained with diverse structures such as highly ordered crystalline forms, non-periodic porous systems, mesoporous amorphous solids (i.e., M41S or SBA-n family supports), non-porous amorphous forms, or totally random structures (Costa et al., 2021 and Zucca and Sanjust, 2014). The natural presence of functional moieties (hydroxyl groups) on the surface of some ceramic materials, such as silica, or the surface preactivation required for other inert surfaces, such as glass or stainless steel, allow the covalent binding of biomolecules (Zdarta et al., 2018).

5.2. Methodologies for Substrate Surface Activation

Surface activation is a set of methods used to alter the chemistry of a substrate surface by introducing chemical groups or charges on the surface. Different techniques are available to make surface modifications to supports prior to immobilization, including silanization as the main strategy (Goddard, 2007). Silanization involves the covering of a substrate surface rich in hydroxyl groups with organo-functional alkoxysilane moieties. Silane-coupling agents, of which 3-aminopropyltriethoxysilane (APTES) is the most representative organosilane, present an alkoxy group and an organofunctional group (amine, thiol, isocyanate, or carboxyl moieties). The alkoxy moiety forms hydrogen bonds with hydroxide groups of the substrate surface, while the organofunctional moiety enables the immobilization of biomolecules that display reactive functionalities, such as amines, carboxylic acids, or aldehydes (Bekmurzayeva et al., 2018). With silica supports, the silanization procedure can be applied during the synthesis of supports (co-condensation functionalization) or after preparing the support (post-synthesis grafting) (Costa et al., 2021). Another strategy to modify the surface reactivity of substrates is biomimetic coating with dopamine. This coating simulates the adhesive properties of marine adhesives by using dopamine that adheres and polymerizes on surfaces, such as metal oxides or polymeric surfaces, although the binding mechanism is not well established (Yah et al., 2012). Once the substrate

surface has been activated, biomolecule immobilization on the material's surface can take place.

5.3. Covalent Immobilization Approaches

For covalent immobilization purposes, the functional moieties of the biomolecule must be compatible with the reactive groups present, either spontaneously or occurring after a previous surface modification on the substrate surface. The biomolecule chemical structure should be studied to preserve antimicrobial properties. The main drawback of covalent immobilization is the potential disruption of antimicrobial activity after bond formation. Immobilized biomolecules can be attached to the surface by a specific site that is responsible for the inhibitory potential, or in a rigid spatial orientation that can significantly change antimicrobial properties. In addition, the grafting process should ensure suitable coating density and uniformity to guarantee the reproducibility and scale up of immobilization, as well as the preservation of the grafted biomolecule's efficient antimicrobial activity (Rosner and Clark, 2021). Therefore, the bonding approach should be carefully designed.

Biomolecules that contain intrinsic chemically reactive groups, such as carboxylic acid, amino, sulfhydryl, and hydroxyl groups, can be covalently immobilized onto activated surfaces through chemical interfacial reactions by non-selective immobilization. This type of grafting can result in more than one type of covalent bonding with different biomolecule orientations (Silva et al., 2016). In contrast, biomolecules that do not possess convenient chemical groups should be modified by diverse chemical strategies. To convert one functional group into another or to assist the grafting process, cross-linking agents, such as glutaraldehyde, carbodiimides, etc., can be used to obtain the needed functional groups, such as thiols, aldehydes, carboxylic acids, hydroxyls, and primary amines (Goddard et al., 2007). The insertion of a specific functional group allows a selective covalent bond to be achieved between the biomolecule and the substrate with a specific conformation (Silva et al., 2016).

5.4. Characterization of Immobilized Antimicrobial Surfaces

The aim of antimicrobial immobilization approaches is to design antimicrobial-coated substrates that should exert their biocidal properties after grafting without apparent release, and result in increased stability, a longer shelf life, and enhanced bioactivity (Ruiz-Rico et al., 2017). To ensure that this goal is fulfilled, a detailed characterization of the developed antimicrobial-coated materials is important to verify the biomolecule's covalent immobilization and to help the reliable interpretation of its antimicrobial mechanism of action after grafting.

Surface chemistry should be characterized by analytical techniques to establish the coating's coverage and the effect of coating on the substrate microstructure, surface charge, surface morphology, porosity, and size (Kazemzadeh-Narbat et al., 2021). These techniques include thermal analyses (i.e., thermogravimetric analysis and differential scanning calorimetry), spectroscopic techniques (i.e., infrared spectroscopy, X-ray spectroscopy, and nuclear magnetic resonance spectroscopy), microscopic techniques (i.e., electron microscopy and scanning microscopy), and other instrumental analyses (i.e., elemental analysis, laser scattering or diffraction analysis, and zeta potential analysis).

The biocidal properties of the immobilized antimicrobial are characterized by in vitro antimicrobial performance testing against microorganisms of interest in different microbial life stages (Ribes et al., 2021a). These viability tests can be combined with microscopic and molecular techniques to reduce errors from viable but non-culturable (VBNC) microorganisms and to help to elucidate the mechanism of action of the grafted antimicrobial (Ribes et al., 2021b). In situ antimicrobial testing is also needed to evaluate biocidal properties in real food matrices, the impact of the immobilized antimicrobial on food properties, and the potential leaching of the grafted biomolecule (Peña-Gómez et al., 2019).

The simulation of the other parameters relevant to the real application of the developed immobilized antimicrobials is another important factor for characterization. Shelf life, cleaning, and stability requirements should be characterized to establish adequate durability (Kazemzadeh-Narbat et al., 2021).

Prior to a real application, the biocompatibility and safety of the immobilized antimicrobials should be characterized by toxicity studies that employ relevant human cell lines and animal models (Fuentes et al., 2021 and Fuentes et al., 2022).

6. Concluding Remarks and Future Perspectives

Seeking new technologies capable of preserving fruit-derived foods and minimizing the impact of processing on the sensory, nutritional, and functional properties of fruit-derived foods, while reducing the carbon footprint of thermal processing, are major challenges for today's food industry. With such tasks, the solution could come from a new approach to the application of natural antimicrobials: their immobilization on food-grade supports. The immobilization of different natural antimicrobials (mostly EOCs) on silica particles is a proven excellent strategy to create effective food preservatives (direct use) or processing aids (indirect use) capable of improving the microbiological quality of fruit-derived foods by extending their shelf life, but without altering nutritional, functional, and sensory properties.

Beyond these advantages, this alternative approach to traditional heat treatment is also seen as an eco-friendly postharvest technology. On the one hand, it could contribute to improving the decarbonization and energy efficiency of the fruit-derived processing sector because it avoids using heat. On the other hand, it provides solutions based on nature, such as natural antimicrobial biomolecules. Moreover, as most of these proposed natural antimicrobials are obtained from plant and animal by-products, their valorization will comply with bioeconomy principles by reducing waste and generating added value to the agri-food sector. Finally, this technology might be used to preserve fruit-derived foods in developing countries with restricted access to other complex and expensive technologies or electricity.

As limitations to the immediate use of this technology, it should be noted that all the applications shown in this work have been exclusively investigated on a laboratory scale, and the immobilization processes described in the different works are laborious and require energy and solvents. These limitations could be reduced

by employing green chemistry to reduce the use of solvents and photocatalysis by resorting to solar or white light as a source of energy for the immobilization steps.

In either case, this review demonstrates how the immobilization of natural antimicrobial compounds on food-grade supports possesses all the features required to be proposed as an eco-friendly postharvest technology that is capable of preserving fruit-derived foods.

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3. Objectives

3. OBJECTIVES

Natural antimicrobials compounds such as essential oil components (EOCs) are effective against a wide range of microorganisms. However, their direct incorporation in food is restricted since i) EOCs change the organoleptic properties of the food, ii) their antimicrobial activity is low in certain food matrices, and iii) their solubility is poor in aqueous media. In response to these drawbacks, different works have proposed the immobilization of EOCs onto the surface of silicon oxide particles. Despite the efforts in the study, development and application of immobilized EOCs, several research gaps have been identified.

Taking this into account, the main objective of this thesis was to develop novel and specific antibacterial and antiviral systems based on the covalent immobilization of essential oil components on food grade supports to be used in the food industry as food preservatives or processing aids for improving the quality and/or extending the shelf life of liquid foods with diverse matrix compositions.

To meet this main objective, the following partial objectives were set:

1. To evaluate the effect of different food components naturally present in food (proteins, lipids, carbohydrates, organic acids, and alcohol) on the antimicrobial activity of essential oil components (EOCs) such as carvacrol, eugenol, geraniol, thymol and vanillin in their free form.
2. To determine the effect of the above-mentioned food components on the antimicrobial activity of vanillin immobilized on silicon oxide particles.
3. To evaluate the effect of free and immobilized forms of eugenol, geraniol, thymol and vanillin on the growth of different strains of the spoilage bacterium *Alicyclobacillus acidoterrestris* in orange juice, as well as the impact on the guaiacol production by the bacteria.
4. To study the antiviral activity of carvacrol, eugenol, thymol and vanillin, both in free and immobilized form against Tulane virus in water.
5. To study consumer perceptions about the use of nanotechnology in the food industry to improve the quality or prolonging the shelf life of foods, among other uses.

4. CHAPTER I
Effect of food matrix components on EOCs antimicrobial activity

4.1. Antimicrobial activity of essential oil components against *Escherichia coli* depends on the food components present in a food matrix

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Abstract

Despite numerous studies evaluating the antimicrobial activity of essential oil components (EOCs) against different microorganisms, the effect of the composition of the matrix in which they are applied remains unexplored. Hence, the effect of different food components (i.e., proteins, lipids, carbohydrates, acids, ethanol) on vanillin antimicrobial activity was carried out by assessing the growth of *E. coli* at different incubation times (0, 1, 4, 8 and 24 h). Based on these outcomes, the food components that most adversely affected vanillin antimicrobial activity were subsequently tested with four other EOCs (i.e., carvacrol, eugenol, geraniol, thymol). The effective concentration of antimicrobials after coming into contact with food components was quantified. The results indicated that bovine serum albumin (BSA), sunflower oil and carbohydrates partially or completely inhibited the antimicrobial efficacy of the tested EOCs, and the inhibition rate depended on the specific EOC-food component combination. Geraniol was notably the most efficient with BSA present. Eugenol performed best with sunflower oil. Carvacrol, eugenol, geraniol and thymol were more effective than vanillin with D-lactose present. This study confirmed that loss of EOCs' effective concentration due to an interaction with food constituents is a significant cause of antimicrobial activity inhibition. These findings underscore the importance of considering matrix composition when selecting antimicrobials to combat a particular strain in real food applications.

Keywords: bovine serum albumin, oil, lactose, antimicrobial effective concentration, killing time

1. Introduction

In today's context, in which a growing number of consumers reject employing synthetic antimicrobial agents as food preservatives, using natural compounds to fight spoilage and pathogenic bacteria has become a significant strategy in food technology. To this end, a great deal of effort has been made to search for natural products that exhibit good bactericidal efficiency. In this regard, essential oil components (EOCs), which are substances produced by aromatic plants as a response to physical or microbiological damage, have become significantly relevant in the field (Amin et al., 2023; Gayán et al., 2020; Hu et al., 2019; Pan et al., 2023). Of the most widely employed EOCs with antibacterial properties, it is worth mentioning vanillin (Va), carvacrol (Car), thymol (Thy), eugenol (Eu) and geraniol (Ger) (Kim and Rhee, 2016).

Vanillin, which is isolated from vanilla seedpods (Noshad et al., 2015), stands out as one of the preferred natural antibacterial agents used in the food industry to extend the shelf life of food and beverage products (Banerjee and Chattopadhyay, 2019). Its popularity lies in its excellent and pleasant aroma, coupled with its proven antimicrobial efficacy against a diverse spectrum of bacterial pathogens, such as *Escherichia coli*, *Listeria monocytogenes,* and *Salmonella enteric,* and fungi, such as *Penicillium* spp., *Alternaria* spp., *Fusarium* spp. (Olatunde et al., 2022). Its antimicrobial activity has been associated with the presence of a phenol and an aldehyde group in its chemical structure (**Figure 1**). Different studies suggest that its antimicrobial action mechanism mainly involves membrane damage and depolarization (Fitzgerald et al., 2004; Lander et al., 2012). Additionally, Va is implicated in reducing ATP content and modulating the expression of crucial genes for bacterial membrane formation (Chen et al., 2023).

Carvacrol, a phenolic monoterpenoid, is extracted mainly from oregano essential oil, whose antimicrobial efficacy is linked with its phenolic moiety and its hydrophobic nature (Kachur and Suntres, 2020) (**Figure 1**). Current research suggests that Car may target the ergosterol biosynthesis pathway by potentially perforating the bacterial membrane (Di Pasqua et al., 2006; Sharifi-Rad et al., 2018).

Extracted predominantly from thyme, Thy is a monoterpenoid whose antimicrobial activity is also associated with the presence of a phenol group on its

structure (**Figure 1**). Thymol causes the disruption or destabilisation of the bacteria cell membrane by inducing changes at intracellular and extracellular ATP levels (de Sousa et al., 2023). This process results in the leakage of intracellular components and the disruption of the proton driving force (Hyun et al., 2020).

Eugenol is the principal constituent of the clove EO, whose antimicrobial activity is associated with the phenol and allyl groups present in its structure (**Figure 1**). This compound is presumed to enhance bacterial membrane permeability by leading to alterations in fatty acid composition (Wang et al., 2018).

Finally, geraniol is a monoterpenoid alcohol that is extracted from geranium plants with antimicrobial activity against different pathogenic bacteria that is associated with its primary alcohol moiety (**Figure 1**). In this case, bacterial cell wall damage is the most accepted explanation for its antimicrobial properties (Albano et al., 2016).

On the whole, these four EOCs also display excellent antibacterial properties against *E. coli, L. monocytogenes* or *S. enterica,* and antifungal properties against *Aspergillus* spp., *Alternaria* spp., *Fusarium* spp. or *Penicillium* spp. (Kalemba and Kunicka, 2003).

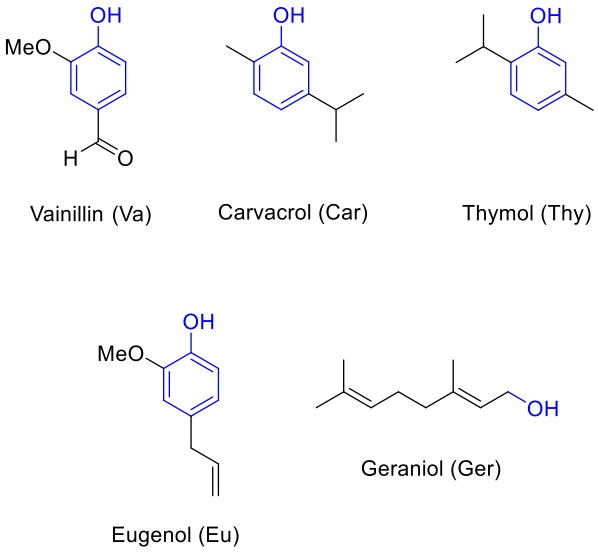

Figure 1. Chemical structures of the essential oil components used in this study. The phenol and alcohol groups of these compounds are highlighted in blue.

Despite the number of *in vitro* studies conducted to investigate the antimicrobial activity of EOCs towards specific bacteria in culture media, *in vivo* data suggest that their activity does not only depend on the EOC-bacteria interaction, but also on the presence of biomolecules in the food system where EOCs operate. For example, Campion et al. (2017) determine that the minimum inhibitory concentration (MIC) for Thy and Car against *E. coli* O157:H7 in Luria-Bertani broth media is 125 µg/mL and 250 µg/mL, respectively. However, when the same concentrations are employed in real food systems, such as milk, the bactericidal effect of these compounds is significantly compromised. In another work, Rattanachaikunsopon and Phumkhachorn (2010) examine the antimicrobial activity of a mixture of cymene (an alkylbenzene related to monocyclic monoterpenes) and Car in both fish broth and carrot juice. The results indicate poorer antimicrobial activity in fish broth, probably due to the presence of fat and proteins in that medium, which would hinder the antimicrobial activity of cymene and Car. When assessing the effect of the *Origanum majorana* L. EO as an antimicrobial agent in sausages, Busatta et al. (2008) report that higher concentrations than the MIC (determined in broth) are required to bring about a bactericidal effect. More recently when evaluating the antimicrobial activity of the *Thymbra capitata* EO, Berdejo et al. (2021) indicate a 2.8-fold increase in the MIC from 250 µL/L in soya tryptone broth to 700 µL/L in skimmed milk. Finally, Coimbra et al. (2022) indicate that the application of MIC×2 of the *Thymus zygis* EO, whose main component is Thy, goes below the quantification limit of bacteria in lettuce model media, but not in chicken juice media. These results generally suggest poorer antimicrobial activity of EOCs in real food systems, probably due to an interaction with proteins, fats and carbohydrates, which would alter their antimicrobial activity.

In addition to these results, which indicate that natural EOCs antimicrobial activity can be modified in real food systems, the influence of the concentration of naturally-occurring food components in certain food has also been addressed. Cava-Roda et al. (2012) report that Va antimicrobial activity is poorer in skimmed milk than in semi-skimmed milk, which would indicate the influence of fat milk content on Va bactericidal efficacy. Smith-Palmer et al. (2001) also report that the effect of different plant extracts (bay, clove, cinnamon and thyme) as natural food preservatives to extend the shelf life of cheese depends on cheese composition.

For low-fat cheese, all the tested oils are able to diminish the microbial population, but only clove is efficient in doing so for full-fat cheese.

According to these pieces of evidence, the present work aims to evaluate the effect of certain food components, such as bovine serum albumin (BSA, protein), sunflower oil (lipid), D-lactose, D-sucrose, pectin and starch (carbohydrates), citric acid (organic acid) and ethanol on the antibacterial activity of Car, Eu, Ger, Thy and Va against *E. coli*. The selection of the aforementioned food components is based on the following assumptions: (i) BSA is a frequently found protein in foods like milk, cheese, yogurt and meat products; (ii) sunflower oil is considered one of the most accepted fatty model systems (Budryn et al., 2014); (iii) D-lactose and D-sucrose are good examples of animal and vegetal disaccharides, while starch and pectin are examples of complex carbohydrates; (iv) citric acid is an organic acid typically present in both fruit (lemons, limes, pineapples, grapefruits, berries) and vegetables (tomatoes, broccoli, carrots, and some pepper varieties); (v) ethanol is present in most fermented alcoholic beverages. This work also pursues to clarify the potential mechanism underlying the reduction in antimicrobial efficacy observed in the presence of specific food components.

2. Materials and methods

2.1. Chemicals

Carvacrol (≥ 98% w/w), eugenol (99% w/w), geraniol (≥ 98% w/w), thymol (≥ 98.5% w/w), vanillin (> 99% w/w), dimethyl sulphoxide (DMSO), BSA, sodium chloride, potassium chloride, monobasic potassium phosphate, pectin and starch were purchased from Sigma-Aldrich (Madrid, Spain). Ethanol, D-sucrose, D-lactose, citric acid, sodium hydroxide, hydrochloric acid, Plate Count Agar (PCA), Trypticase Soya Agar (TSA), Tryptone Soya Broth (TSB) and methanol (HPLC grade) were supplied by Scharlab (Barcelona, Spain). Sunflower oil was purchased in a local supermarket.

2.2. Microbiological assays

2.2.1 Bacteria culture

The *Escherichia coli* K12 strain employed in this study was provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). This bacterial strain was firstly reconstituted from cryovials by seeding in PCA and incubating at 37 °C overnight, followed by incubation in TSB at 37 °C overnight. PCA and TSB were prepared following the manufacturer's instructions.

2.2.2 Food components

The concentration of the food components employed in this work was based on the standard composition in the proteins, fat, carbohydrates, organic acids and alcohol of some of the most consumed liquid foods (milk, beer, juice, wine), and also on the information available in different scientific works, legislation and international food databases (Cava-Roda et al., 2012; European Parliament and Council, 2013; USDA, 2023). The following concentrations were used: (i) BSA: 0.5, 1.5 and 3 g/100 mL; (ii) sunflower oil: 1, 2 and 4 g/100 mL; (iii) D-lactose: 1.5, 3 and 4.5 g/100 mL; (iv) D-sucrose: 5, 8 and 12 g/100 mL; (v) pectin: 0.1, 0.5 and 1 g/100 mL; (vi) starch: 0.1, 1 and 2 g/100 mL; (vii) citric acid: 0.1, 0.2 and 0.3 g/100 mL; (viii) ethanol: 2, 6 and 12 g/100 mL.

Each component was dissolved in Phosphate Buffered Saline (PBS) solution, prepared by mixing 136.9 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium phosphate heptahydrate and 1.5 mM potassium phosphate monobasic in distilled water. pH was then adjusted to 7.4 using 1 M HCl (Sahakijpijarn et al., 2019). The resulting component solutions were filtered and inoculated with *E. coli* to reach a final concentration of 5 $\log_{10}$CFU/mL (5 $\log_{10}$).

2.2.3 Susceptibility studies

The microdilution method was followed to determine the MIC of each food component against *Escherichia coli* K12 (Chandrasekaran and Venkatesalu, 2004; Kirchhoff et al., 2018). The MIC values were defined as the lowest concentration at which bacterial growth was no longer evident. The DMSO stock solutions (300 mg/mL) of Car, Eu, Ger, Thy and Va were employed. For assays, aliquots of stock

solutions were prepared by dilution from the stock in TSB medium to obtain concentrations of 2, 1.8, 1.6, 1.4, 1.2, 1, 0.8, 0.6, 0.4, 0.2 mg/mL. Bacterial suspensions (9 $\log_{10}$) were transferred to plates to obtain a final inoculum of 5 $\log_{10}$. Positive (bacteria in TSB and in TSB with 1% DMSO) and negative (TSB and TSB with 1% DMSO) controls were also included. The quoted results are the mean of three independent replicates in triplicate (n=9).

2.2.4 Time-kill curve studies

To quantify the impact of the selected matrix components on the bactericidal activity of EOCs (Car, Eu, Ger, Thy, Va), time-kill curves were applied for *E. coli*. This methodology allows not only the bactericidal efficacy of the selected agents to be evaluated, but also provides information about the dynamics of this action. During a typical assay, bacterial cultures were set up using MIC×0 (control), ×1 and ×2, and were incubated at 37 °C (see Section 2.2.3 for details). All the assays were performed using 30 mL of inoculated food media. The viability of *E. coli* cells after treatment with EOCs was quantified by the microdilution method at different incubation times (0, 1, 4, 8 and 24 h). For this purpose, 1 mL of each sample was serially diluted and plated in TSA colonies, which were counted after 24 h of incubation at 37 °C. Time-kill curves were obtained by plotting the $\log_{10}$ of bacteria against time (Xedzro et al., 2022). Three independent analyses were carried out (n=3).

2.3. Analysis of the effect of food components on the EOCs concentration

An HPLC analysis was performed to determine the Car, Eu, Ger, Thy and Va concentrations in the presence of a selection of food components that covered each group of food components and that inhibited EOCs' antimicrobial activity the most. To this end, the samples containing each of the combinations of MIC×1 of Car, Eu, Ger, Thy or Va and BSA (3 g/100 g), sunflower oil (4 g/100 g) or lactose (4.5 g/100 g) in PBS were prepared. Samples of each EOC in the absence of food components were employed as the negative controls. Samples were then incubated at 37 °C. After different incubation periods (0, 1, 4, 8 and 24 h), 1 mL of each sample was filtered and analysed by HPLC according to the method described by Pérez-Esteve et al. (2016) with minor modifications. The HPLC analysis was

performed in a Hitachi LaChrom Elite HPLC system (Hitachi Ltd., Tokyo, Japan), equipped with an auto-sampler (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 x 4.6 mm) was used. A flow rate of 1.0 mL/min at 25 °C and an injection volume of 10 µL were applied. For Va detection, elution started with a linear gradient of 50-100% of B for 2 min, followed by an isocratic elution with 100% of B for 3 min [A = deionised water (Aquinity deionizer, Membrapure GmbH, Berlin, Germany); B = methanol]. Then the elution conditions were returned to 50% B for 2 min, and finally isocratic conditions were maintained for 5 min. The wavelength of UV detection was set at 231 nm. For the Car, Eu, Ger and Thy analyses, the latter chromatographic conditions also applied, but the wavelength of the UV detector was set at 275, 280, 210 and 277 nm, respectively. The HPLC analyses were done in triplicate (n=3).

2.4. Statistical analysis

For each different assay, data were statistically analysed by a multifactor analysis of variance (multifactor ANOVA). The LSD (least significant difference) procedure was used to test the differences with a 95% confidence interval ($p<0.05$) using Statgraphics Centurion XVIII (Statpoint Technologies, Inc., Warrenton, VA, USA).

3. Results and discussion

3.1 Impact of the assayed food components on *E. coli* growth

Firstly, the effect of the food components employed in this study on *E. coli* growth was assayed by the time-killing curves approach. **Figure 2** shows *E. coli* growth in PBS in both the presence and absence of the different food components at MIC×0. The results revealed that the addition of increasing concentrations of BSA, sunflower oil and some carbohydrates (D-lactose, D-sucrose or starch) allowed the bacterial population to grow up to *ca*. 1 $\log_{10}$ in relation to the control (PBS). On the contrary, addition of citric acid or ethanol led to a reduction in > 5 $\log_{10}$ after 8 h of incubation at concentrations of up to 0.2 g of citric acid/100 mL or 6 g ethanol/100 mL. Overall, these results confirmed both the need for a carbon source

for *E. coli* growth (Tong et al., 2020) and the bactericidal properties of citric acid and ethanol (Molina et al., 2003). Based on these results, the maximum concentrations of citric acid and ethanol were set at 0.2 g and 12 g/100 mL, respectively.

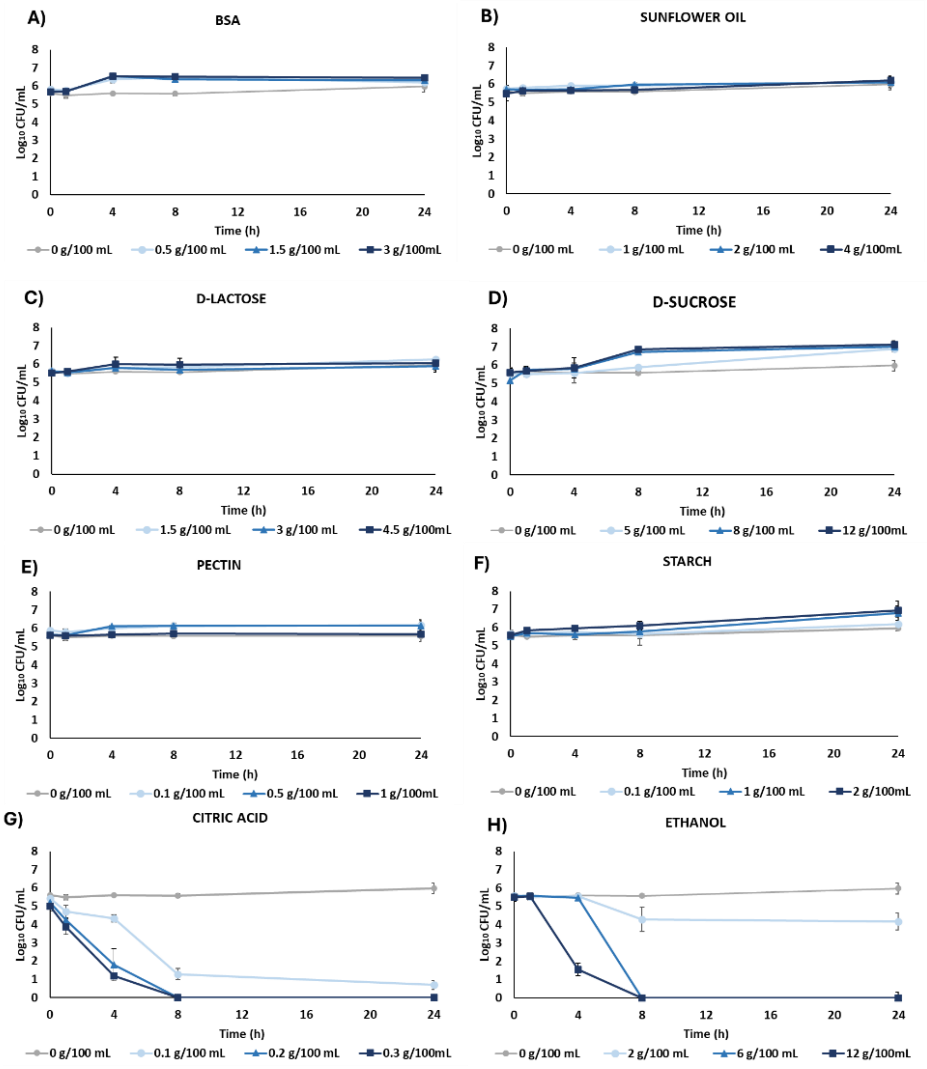

Figure 2. Time-kill curves for *E. coli* treated with bovine serum albumin (BSA) (A), sunflower oil (B), D-lactose (C), D-sucrose (D), pectin (E), starch (F), citric acid (G), and ethanol (H) in absence of essential oil components.

3.2 Effect of the assayed food components on vanillin bactericidal efficacy

After characterising *E. coli* growth on each medium, the effect of adding different antimicrobials was tested. Based on its extended use in the food industry, Va was firstly selected to assay the impact of food components on antibacterial activity. To this end, the MIC value of Va against *E. coli* in PBS was firstly determined. It was 2 mg/mL, a value that agrees with the data previously reported by Chen et al. (2023) and comes close to 2.5 mg/mL described by Yuan et al. (2019). Next the time-kill curves of the *E. coli* treated with Va were determined for each food component at two concentrations: MIC×1 (2 mg/mL) and ×2 (4 mg/mL) (**Figure 3**). The results are discussed below.

3.2.1 BSA and sunflower oil

As shown in **Figure 3A**, Va antimicrobial activity was significantly inhibited by the presence of BSA after 24 h of incubation and at all tested protein concentrations. Despite doubling the Va concentration (MIC×2), suppressed antimicrobial activity persisted at the protein concentrations of 1.5 or 3 g BSA/100 mL. Only at the lowest tested BSA concentration (0.5 g BSA/100 mL) was a marginal recovery in bacterial reduction of approximately 1 $\log_{10}$ observed. These results state the good capacity of BSA to inhibit Va antimicrobial capacity.

With sunflower oil, Va antimicrobial activity at MIC×1 was hindered at all the tested concentrations, and was reinstated by doubling the Va concentration (MIC×2) ($p<0.001$) (**Figure 3B**). In the latter case however, the time required to reduce bacterial growth below the limit of quantification, also known as killing time (Li et al., 2013), increased compared to the control (lack of lipids). This observed killing time delay suggests that despite achieving complete *E. coli* growth inhibition after 24 h of incubation with Va, the presence of fat alters intrinsic Va antimicrobial properties.

3.2.2 D-lactose, D-sucrose, pectin and starch

The outcomes of the studies performed in the presence of simple sugars showed that D-lactose partially hindered Va antibacterial activity, which was remarkable at the MIC×1 concentration (**Figure 3C**). Although a sharp drop of > 5 $\log_{10}$ in *E. coli* was accomplished in PBS after 24 h of incubation, this effect in the presence of D-lactose lowered to 2.5-fold (2 $\log_{10}$) regardless of the tested concentration (from 1.5 to 4.5 g D-lactose/100 mL). For sunflower oil, the bactericidal effect was restored by doubling the Va concentration (MIC×2) but, in this case, the killing time was also delayed to 8 h at all the tested D-lactose concentrations.

Similar results were obtained in the assays performed in the presence of D-sucrose (**Figure 3D**). At the MIC×1 concentration however, the impact of D-sucrose on Va antimicrobial activity appeared to be slightly less pronounced compared to D-lactose, with a reduction in bacterial viability of approximately 2.5 $\log_{10}$ after 24 h of incubation. As before, this effect was avoided by doubling the Va concentration (MIC×2) and a killing time delay was also noted.

With pectin, at the Va MIC×1 concentration, the addition of 0.1, 0.5 and 1 g pectin /100 mL to growth media had a distinct impact on Va bactericidal efficacy ($p<0.05$) (**Figure 3E**). Surprisingly, the strongest inhibition effect occurred at the lowest tested pectin concentration (0.1 g pectin/100 mL), with 2 $\log_{10}$ of bacteria counts after 24 h of incubation. In contrast, when employing 0.5 or 1 g pectin/100 mL and MIC×1 of Va, bacterial growth completely reduced after 24 or 4 h of incubation, respectively. The positive synergy effect of high pectin concentrations on Va antimicrobial activity might be due to the formation of hydrogels, which could facilitate the internalisation of this antimicrobial compound into the bacterium (Yang et al., 2020). As before, doubling the Va concentration (MIC×2) completely reduced cell viability after 8 h of incubation in 0.1 g pectin/100 mL and shortened the killing time from 24 h to 4 h in 0.5 g pectin/100 mL.

Finally, using starch partially inhibited Va antibacterial activity (MIC×1 concentration), whose impact relied on starch content ($p<0.001$) (**Figure 3G**). At the MIC×1 concentration of Va, bacterium growth reduction of ca. 2.5 $\log_{10}$ at 0.1 g/100 mL of starch was observed after 24 h of incubation. This activity decreased as the starch concentration in the medium increased so that the higher the starch

concentration, the poorer Va antimicrobial efficacy was, with an almost 80% loss of its antimicrobial activity at the 2 g of starch/100 mL concentrations. Once again, doubling the Va concentration (MIC×2) completely lowered bacterial counts (> 5 $\log_{10}$) after incubating for 24 h in any starch medium. However, the presence of starch provoked a killing time delay.

In short, our results reveal that the presence of certain concentrations of D-lactose, D-sucrose, pectin and starch reduces Va intrinsic antibacterial activity (MIC = 2 mg/mL). Thus applying higher concentrations of this natural antimicrobial is necessary to restore the effect. Of the tested carbohydrates, D-lactose had the most marked negative impact on Va antibacterial activity, which was avoided by doubling the Va concentration (4 mg/mL). The distinct impact of the tested carbohydrates on Va activity might be explained by their different structural architecture (monosaccharide composition and linkage, overall arrangement, size), which might be a key point when interacting with Va. So besides D-lactose and D-sucrose both being disaccharides, the former is a reductive sugar composed of a β-D-galactopyranoside and a D-glucopyranoside unit linked through positions (1–4), while the latter is a non-reductive sugar containing an α-D-glucopyranoside unit linked by its anomeric position to a β-D-fructopyranoside, which is a five-membered monosaccharide (Plazinski et al., 2016). Pectin and starch are both large carbohydrates and polysaccharides that result by the polymerisation of D-galacturonic acid and D-glucose, respectively. Whereas D-lactose, D-sucrose and starch are neutral carbohydrates whose structure contains mainly hydroxyl groups and are able to interact by hydrogen-bonding with Va, pectin is a negatively charged carbohydrate because its structure contains carboxylate groups. All these relevant structural differences might explain the distinct effects caused by these food components on Va bactericidal activity.

3.2.3 Citric acid and ethanol

Unlike BSA, with sunflower oil and the studied carbohydrates, which all had a negative impact on Va antibacterial activity, the presence of both citric acid and ethanol had a positive synergic effect on Va antibacterial activity (**Figure 3H**).

The assays using 0.1 or 0.2 g/100 mL of citric acid and MIC×1 of Va showed the total inhibition of the Va antibacterial effect. When the citric acid concentration was increased to 0.3 g/100 mL, complete inhibition along with a shorter killing time occurred. Doubling the Va concentration (MIC×2) caused slightly greater antimicrobial activity ($p<0.05$), although the killing time was the same compared to the MIC×1 application for all the citric acid concentrations. The assays carried out in the presence of different quantities of ethanol also showed a positive synergistic effect along with a shorter killing time (**Figure 3I**).

The synergistic effect of citric acid or ethanol on Va antimicrobial activity might be due to both food components' ability to affect the parameters directly related to improvements in compound antimicrobial activity, such as solubility or bacterial cell internalisation, among others (Da Cruz et al., 2022; Ingram 1981; Negi, 2012).

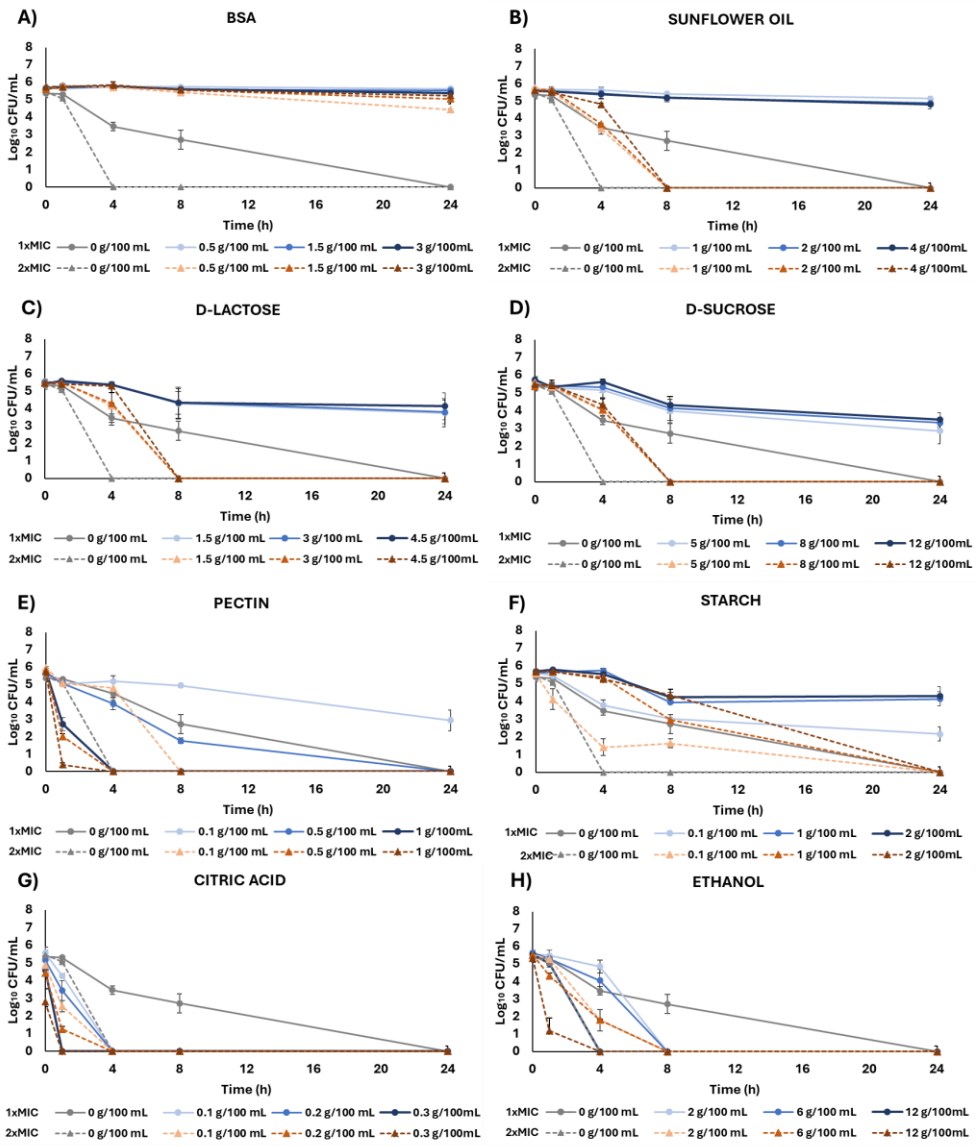

Figure 3. Time -kill curves for *E. coli* treated with MIC×1 (continuous blue lines) and MIC×2 (dashed orange lines) concentrations of vanillin (Va) and in presence and in the absence of diverse concentrations of bovine serum albumin (BSA) (A), sunflower oil (B), D-lactose (C), D-sucrose (D), pectin (E), starch (F), citric acid (G) and ethanol (H).

3.2.4 Effect of single or dual factors on Va antimicrobial inhibition

After quantifying the individual effect of food components on *E. coli* growth, multifactorial statistical analyses were performed for each food component to identify the most significant factors responsible for the observed Va antimicrobial inhibition. As outlined in ***Table S1***, the effect of the Va concentration (C) and incubation time (t) gave the lowest p-value and the highest F-ratio and were, thus, the most important factors that affect the reduction in bacteria growth. On the contrary, the concentration of some food components, such as sunflower oil, D-lactose and D-sucrose, did not significantly alter Va bactericidal efficacy, probably because these compounds at very low concentrations were already able to hinder Va antimicrobial activity. In a deeper analysis, assessing dual interactions revealed prevalent interdependence among factors. Specifically, the interaction labelled as C×t obtained the lowest p-value and the highest F-ratio, which indicate that the effect of the EOC concentration very much depends on the incubating time. This analysis unequivocally confirms a significant effect of EOC concentration, incubation time and compound concentration, as well as dual interactions, and indicates an interactive effect among these variables.

Table *S1*. F-ratio and p-value, obtained in the multifactor ANOVA for *E. coli* growth after treatment with vanillin in the presence of different food components. BSA: bovine serum albumin; EOC: essential oil component.

Compound	Factor	F-ratio	p-value
	C: EOC concentration	28.22	0.00
	t: incubation time	77.51	0.00
	c: Compound concentration	4.15	0.0197
BSA	**INTERACTIONS**		
	C×t	26.45	0.00
	C×c	10.23	0.0001
	t×c	1.70	0.1338
	C: EOC concentration	6622.87	0.00
	t: incubation time	1844.93	0.00
	c: Compound concentration	2.91	0.0609
Sunflower oil	**INTERACTIONS**		
	C×t	1282.49	0.00
	C×c	19.25	0.00
	t×c	9.40	0.00
	C: EOC concentration	370.11	0.00
	t: time of incubation	245.85	0.00
	c: Compound concentration	1.04	0.3597
D-Lactose	**INTERACTIONS**		
	C×t	82.18	0.00
	C×c	0.16	0.8512
	t×c	0.45	0.8432
	C: EOC concentration	726.93	0.00
	t: incubation time	566.96	0.00
	c: Compound concentration	3.17	0.6512
D-Sucrose	**INTERACTIONS**		
	C×t	144.92	0.00
	C×c	1.03	0.3616
	t×c	0.36	0.9032

Table *S1*. Continuation

Compound	Factor	F-ratio	p-value
	C: EOC concentration	84.02	0.00
	t: incubation time	53.64	0.00
	c: Compound concentration	106.12	0.00
Pectin	**INTERACTIONS**		
	C×t	2.32	0.0816
	C×c	8.52	0.00
	t×c	6.77	0.00
	C: EOC concentration	527.59	0.00
	t: incubation time	627.42	0.00
	c: Compound concentration	289.51	0.00
Starch	**INTERACTIONS**		
	C×t	126.28	0.00
	C×c	9.79	0.00
	t×c	26.89	0.00
	C: EOC concentration	97.33	0.00
	t: incubation time	861.11	0.00
	c: Compound concentration	237.07	0.00
Citric acid	**INTERACTIONS**		
	C×t	97.83	0.00
	C×c	25.47	0.00
	t×c	237.07	0.00
	C: EOC concentration	17.69	0.00
	t: incubation time	107.23	0.00
	c: Compound concentration	18.04	0.00
Ethanol	**INTERACTIONS**		
	C×t	5.90	0.00
	C×c	0.05	0.5732
	t×c	6.59	0.00

3.3 Effect of diverse food components on carvacrol, eugenol, geraniol, and thymol bactericidal efficacy

The results presented in Section 3.2 clearly demonstrate that Va antibacterial activity is modified by the presence of diverse food components. In particular, the impact of BSA, sunflower oil and D-lactose is especially noteworthy, even at low concentrations. These outcomes led us to explore whether this inhibition against *E. coli* is exclusive for Va or if it could also occur for other antimicrobial agents widely employed in the food industry, specifically phenols like Car, Eu or Thy, or alcohols like Ger.

For this assessment, the MIC values of Car, Eu, Thy, and Ger against *E. coli* were firstly determined. The results showed that Car, Eu, Ger and Thy exhibited greater antimicrobial activity against *E. coli* than Va. Car and Thy proved the most potent compounds with MIC values of 0.20 mg/mL, followed by Eu and Ger with MIC values of 0.6 and 0.8 mg/mL, respectively. These values are in agreement with previously reported data. Corona-Gomez et al. (2022) found MICs ranging from 0.02 to 0.2 mg/mL when testing thymol or carvacrol against gram-negative bacteria, including *E. coli* ATCC 8739. Cava-Roda et al. (2021) obtained a MIC of eugenol of 0.766 mg/mL in *E. coli* O157:H7. The time-kill curves for the *E. coli* treated with Car, Eu, Ger and Thy at the two different concentrations (MIC×1 and ×2) were then determined. For Car, Eu and Thy at MIC×1, 4 h of incubation were required to totally reduce bacterial growth, while only 1 h was needed for Ger at the same concentration. As expected, the killing time dropped to 1 h for all cases when the concentration of these EOCs was doubled. Considering these results, the time-kill curves for the *E. coli* treated with Car, Eu, Ger and Thy in the presence of BSA, sunflower oil and D-lactose were then measured, which are discussed below.

3.3.1 BSA

Figure 4 shows Car, Eu, Ger and Thy antimicrobial activity in the presence of BSA. At the MIC×1 concentration of Car, Eu and Thy, and at 0.5 g/100mL of BSA, the three EOCs had a bactericidal effect on *E. coli* after 24 h of incubation ($p<0.05$). By increasing the BSA concentration to 1.5 g/100 mL, these three components displayed bacteriostatic behaviour. However, this effect was lost in the presence of 3 g/100 mL BSA, where *E. coli* was able to grow. On the contrary, a bactericidal effect was observed for Ger under all the tested conditions, which suggests that the protein concentration influenced only the killing time ($p<0.05$). The higher the protein concentration, the longer the killing time, with microbial counts below the detection limit for incubation times longer than 8 h. At the MIC×2 concentration, the presence of 1.5 or 3 g BSA/100 mL also inhibited Car, Eu and Thy antimicrobial activity, but full *E. coli* growth inhibition occurred in less than 4 h for 0.5 g BSA /100 mL.

Taken together, these results revealed that Va was the antimicrobial compound most influenced by the presence of BSA, closely followed by Car and Thy, and lastly by Eu. In these media, Ger activity did not alter, not even at the highest protein concentration. Therefore, the use of Car, Eu, Thy and Va should be avoided to extend the shelf life of protein-rich products like milk or soya milk, where the protein concentration is approximately 3 g/100 mL (USDA, 2023). For the latter products, despite Ger having a higher MIC value than Car or Thy, it would be the most appropriate bactericidal agent because the presence of BSA did not alter its activity. In contrast, the use of Car, Eu, Thy would be adequate for products like red wine or beer, for which the protein concentration is rather low: 0.07 and 0.46 g protein/100 mL, respectively (USDA, 2023).

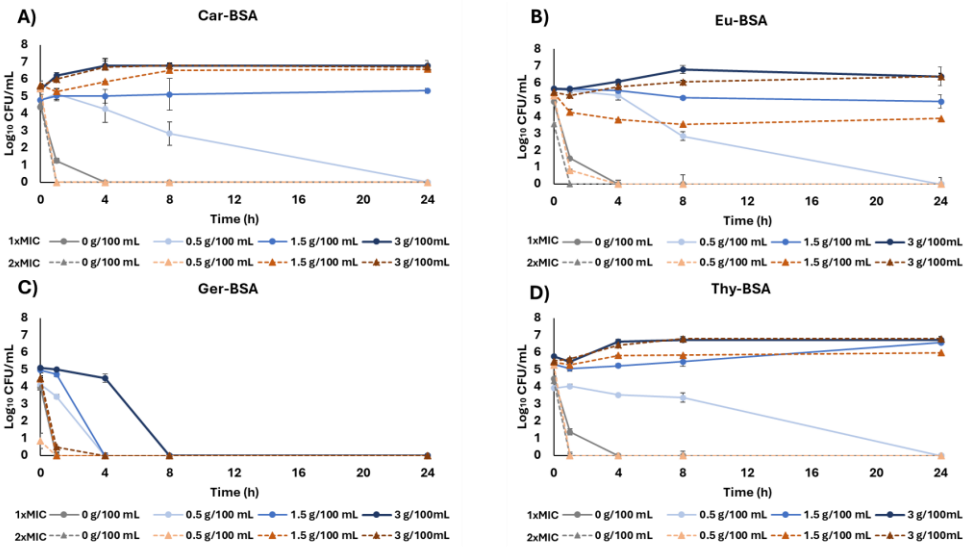

Figure 4. Time-kill curves for *E. coli* treated with MIC×1 (continuous blue lines) and MIC×2 (dashed orange lines) concentrations of carvacrol (Car) (A), eugenol (Eu) (B), geraniol (Ger) (C) and thymol (Thy) (D), in presence and in the absence of diverse concentrations of bovine serum albumin (BSA).

3.3.2 Sunflower oil

Figure 5 shows the impact of sunflower oil on Car, Eu, Ger, and Thy antimicrobial effectiveness by revealing three distinct patterns. The behaviour of Car and Thy was similar ($p>0.05$), for which antimicrobial activity was totally inhibited at all the tested sunflower oil concentrations, regardless of the applied EOCs concentration (MIC×1 or ×2). The second was Ger, which at MIC×1 reduced the microbial load between 1-2 cycles after 24 h of incubation depending on the lipid concentration ($p<0.05$). The higher the lipid concentration, the less marked the reduction. On the contrary, by doubling its concentration to MIC×2, the bacterial population (>5 $\log_{10}$) totally diminished at all the sunflower oil concentrations after 8 (1 g/100 mL) or 24 h (> 2 g/100 mL) of incubation. Finally for Eu, the application of MIC×1 or ×2 completely removed *E. coli* after 24 h of incubation at all the tested sunflower oil concentrations, although there were statistical differences among lipid concentration ($p<0.05$). Employing MIC×2 resulted in a reduction of up to 4 $\log_{10}$

within the first 4 h of incubation *versus* the 1 log_{10} reached at MIC×1. Despite this fact, the killing time did not differ between both Eu concentrations.

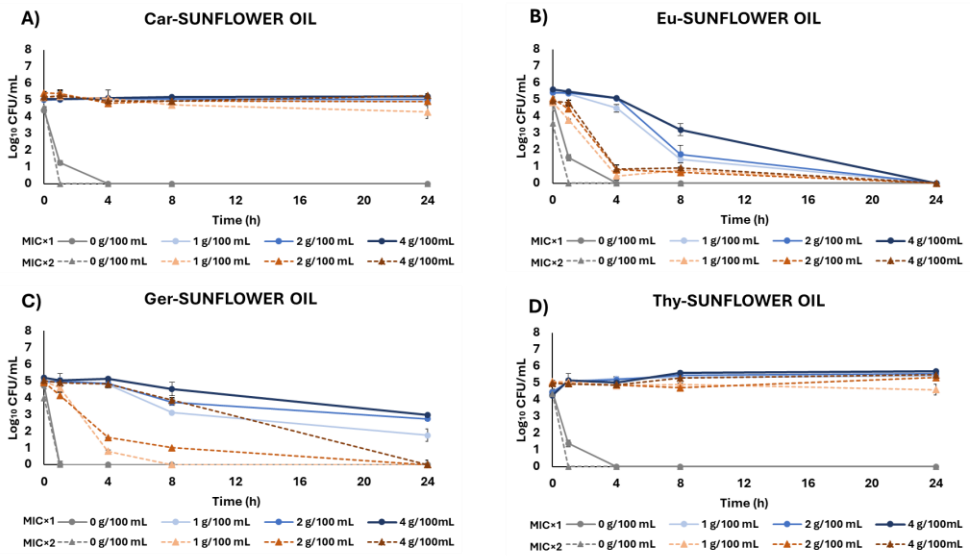

Figure 5. Time-kill curves for *E. coli* treated with MIC×1 (continuous blue lines) and MIC×2 (dashed orange lines) concentrations of carvacrol (Car) (A), eugenol (Eu) (B), geraniol (Ger) (C) and thymol (Thy) (D), in presence and in the absence of diverse concentrations of sunflower oil.

Taken together, Car and Thy would be ineffective antibacterial agents for controlling *E. coli* in products like semi-skimmed milk, whole milk or vegetable-derived beverages because the total lipids concentration of these products is usually high: about 1.9 g /100 mL, 3.2 g/100 mL and 2.5 g/100 mL, respectively (USDA, 2023). On the contrary for products like red wine or beer, employing any of the herein evaluated EOCs would be adequate because these products' fat content is negligible (USDA, 2023). In juice, where the reported lipid concentration is also low (about 0.34 g/100 mL), utilising Eu or Ger would be recommended.

3.3.3 D-lactose

The effect of D-lactose on Car, Eu, Ger and Thy antimicrobial activity is shown in **Figure 6**. Unlike Va, whose antibacterial activity was significantly inhibited in the presence of D-lactose, no relevant variations were observed at the concentration of either MIC×1 or ×2 of Car, Eu, Ger and Thy, and total *E. coli* removal was accomplished in all cases ($p>0.05$). For all the assayed D-lactose concentrations however, the killing time was 4 h for all the EOCs studied at MIC×1, while the increase in the EOCs concentration to MIC×2 shortened the killing time to 1 h ($p<0.05$). Based on the herein reported results, and given D-lactose content, Va should be replaced with Car, Eu, Ger or Thy in dairy products (i.e., hot chocolate drinks, malted milk, milk, smoothies) or products containing more than 1.5 g/100 mL D-lactose (USDA, 2023) to control *E. coli* contaminations in these foods. Nevertheless, Eu or Ger would be the most suitable ones if these products are rich in lipids or proteins.

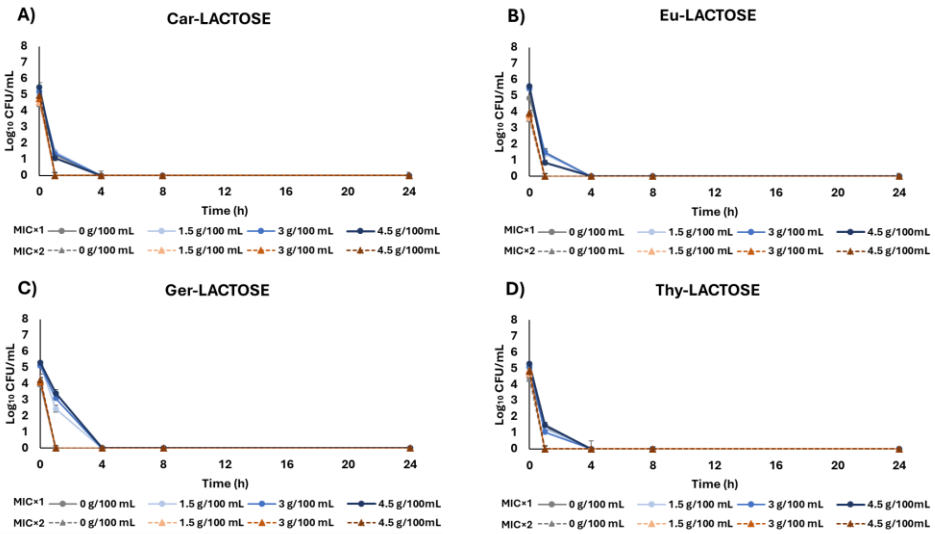

Figure 6. Time-kill curves for *E. coli* treated with MIC×1 (continuous blue lines) and MIC×2 (dashed orange lines) concentrations of carvacrol (Car) (A), eugenol (Eu) (B), geraniol (Ger) (C) and thymol (Thy) (D), in presence and in the absence of diverse concentrations of D-lactose.

3.3.4. Effect of a single factor or dual factors on EOCs antimicrobial inhibition

After evidencing the different antimicrobial hindering behaviours exhibited by the five studied antimicrobials in the presence of BSA, sunflower oil and D-lactose, multifactorial statistical analyses were conducted for all three food components. The aim was to identify the most significant factors, or the dual factor combinations, responsible for the observed antimicrobial inhibition. As seen in ***Table S2***, for BSA and sunflower oil all the study variables and their interactions exhibited a statistically significant influence on *E. coli* growth. For all the factors and dual interactions, the p-value was <0.05. Regarding the F-ratios analysis, the BSA concentration (for BSA) and the EOC concentration (for sunflower oil) were the variables with the most weight for the effect of antimicrobials on lowering *E. coli* counts. For D-lactose, the observed differences were only statistically relevant ($p<0.05$) for the single factors and some dual interactions. Regarding the F-ratio, the incubation time was the factor with the heaviest weight on the response variable (*E. coli* growth), as **Figure 6** clearly evidenced.

Table S2. F-ratio and p-value, obtained in the multifactor ANOVA for *E. coli* growth after treatment with carvacrol, eugenol, geraniol, thymol, and vanillin in the presence of BSA, sunflower oil and D-lactose. BSA: bovine serum albumin; EOC: essential oil component.

Compound	Factor	F-ratio	P-value
	T: EOC type	3860.23	0.00
	C: EOC concentration	3895.80	0.00
	t: Incubation time	53.96	0.00
	c: Compound concentration	6517.16	0.00
	INTERACTIONS		
BSA	**T×C**	510.25	0.00
	T×t	86.22	0.00
	T×c	899.55	0.00
	C×t	35.87	0.00
	C×c	582.26	0.00
	t×c	125.28	0.00
	T: EOC type	2873.80	0.00
	C: EOC concentration	4177.27	0.00
	t: incubation time	2511.49	0.00
	c: Compound concentration	272.52	0.00
	INTERACTIONS		
Sunflower oil	**T×C**	755.21	0.00
	T×t	447.65	0.00
	T×c	60.43	0.00
	C×t	318.80	0.00
	C×c	24.08	0.00
	t×c	30.68	0.00
	T: EOC type	3108.46	0.00
	C: EOC concentration	1347.42	0.00
	t: incubation time	6818.40	0.00
	c: Compound concentration	5.74	0.0035
	INTERACTIONS		
D-Lactose	**T×C**	179.35	0.00
	T×t	274.71	0.00
	T×c	0.75	0.6484
	C×t	85.26	0.00
	C×c	0.35	0.7038
	t×c	1.43	0.1811

3.4. Effect of food components on EOCs concentration

Previous sections confirmed that the bactericidal efficacy of the five EOCs employed in this work against *E. coli* is altered by the presence of certain usual food components, and the type and magnitude of antimicrobial inhibition differs for each EOC-food component combination. These results highlight the complexity of predicting the efficacy of EOCs against a certain microbial strain and underline the need to understand how food matrices impact antimicrobial outcomes. One possible hypothesis for this antimicrobial activity inhibition would be the drop in the effective concentration of the antimicrobial in the medium provoked by possible interactions between EOCs and food components. To explore this hypothesis, the concentrations of the Car, Eu, Ger, Thy and Va remaining in media after exposure to BSA, sunflower oil and D-lactose was analysed by HPLC with a UV detector.

Table 1 shows the effective concentration EOCs after incubating MIC×1 of these compounds with the highest tested concentrations of BSA, sunflower oil or D-lactose (3, 4 and 4.5 g/100 mL, respectively) at different incubation times (0, 1, 4, 8 and 24 h). For most of the studied conditions, no relevant differences were found when comparing the EOC concentration after incubation in either the absence or presence of food components (**Table 1**). However, in some cases there was an immediate interaction between EOCs and food components, which continued throughout the study period.

In particular, the incorporation of BSA into bacterial growth media led to a 70-fold reduction in the initial Va concentration, possibly due to the formation of Schiff-base species resulting from the reaction between the aldehyde group of Va and the ε-amino group of the lysine residues of BSA (Bashiri et al., 2021). Of all the herein studied EOCs, Va was the only compound whose structure contained this electrophilic functional group (**Figure 1**). In the protein, thus reducing its concentration in the free form to interact with the bacteria, reducing its bactericidal efficacy. This phenomenon has already been reported by Chobpattana et al. (2002), who describe a drop of ca. 30% in the Va concentration in media containing 3 g BSA/100 mL, and the formation of BSA-Va adducts may be a possible mechanism for inhibiting antibacterial activity.

For Ger, the effective EOC concentration modestly lowered (20-fold), which suggests that this compound is also capable of interacting with the protein, but more weakly and without involving covalent linkage. As Ger is a primary alcohol in this case, it could promote hydrogen bonding interactions between its hydroxyl group and the acceptor groups in BSA (i.e. the main carbonyl groups), along with diverse lipophilic interactions between its carbon skeleton and the side chain of the non-polar residues in the protein pocket.

On the contrary, despite the bactericidal activity of Car, Eu or Thy being partially reduced in the presence of BSA, no significant differences were identified in these compounds' free concentration, which implies that this inhibition would go beyond the drop in the effective EOCs concentration.

Regarding the interaction with sunflower oil, the results showed that the reduction in the free concentration for Thy, Car, Eu and Va was 80-, 70-, 16- and 4-fold, respectively (**Table 1**). No difference in the free concentration ($p>0.05$) was found for Ger. The markedly distinct behaviour of Thy and Car might be explained by their intrinsically higher hydrophobicity compared to Eu and Va (Ben Arfa et al., 2006). This fact was evidenced by the comparison made of the LogP (P=partition coefficient) values of all these compounds because P is a parameter that is directly related to the compound lipophilicity. According to the data retrieved from PubChem (Kim et al., 2016), Car and Thy show LogP values of 3.1 and 3.3, respectively, but the Eu and Va values are lower, respectively with 2.0 and 1.2. Therefore, as Car and Thy would have a higher affinity for lipidic systems, such as sunflower oil, than Eu and Va, this would reduce their free concentration in aqueous media to a greater extent and, consequently, their antimicrobial activity. Moreover, although the 10-carbon chain of Ger resulted in lower solubility in aqueous media (LogP = 2.9) compared to other shorter-chain alcohols (i.e., ethanol), its aliphatic structure would result in a different interaction with lipids than that of other investigated aromatic EOCs. At the same time, the minor reduction in the Ger and Eu concentrations could explain why these compounds' antimicrobial activity continued (see Section 3.3.2).

With D-lactose, no significant changes ($p>0.05$) in the effective EOCs concentration after adding the food component were found. This implies that the

experimentally observed decline in Va bactericidal efficacy may derive from factors not related to those elucidated for BSA and sunflower oil.

Table 1: Free concentration (mg/mL) of carvacrol (Car), eugenol (Eu), geraniol (Ger), thymol (Thy) and vanillin (Va), in the presence and in the absence of bovine serum albumin (BSA), sunflower oil and D-lactose in the growth media at different incubation times. EOC: essential oil component

Food constituent	EOC	g/100 mL food constituent	Incubation Time (h)				
			0	1	4	8	24
BSA	Car	0	0.19 ± 0.02	0.18 ± 0.03	0.18 ± 0.02	0.20 ± 0.01	0.19 ± 0.02
		3	0.18 ± 0.03	0.18 ± 0.03	0.17 ± 0.03	0.18 ± 0.03	0.18 ± 0.03
	Eu	0	0.59 ± 0.08	0.58 ± 0.09	0.55 ± 0.06	0.56 ± 0.09	0.55 ± 0.06
		3	0.58 ± 0.06	0.57 ± 0.06	0.54 ± 0.04	0.55 ± 0.03	0.53 ± 0.05
	Ger	0	0.79 ± 0.06^{a}	0.78 ± 0.01^{a}	0.79 ± 0.09^{a}	0.79 ± 0.09^{a}	0.79 ± 0.10^{a}
		3	0.64 ± 0.06^{b}	0.64 ± 0.08^{b}	0.64 ± 0.03^{b}	0.64 ± 0.08^{b}	0.65 ± 0.06^{b}
	Thy	0	0.21 ± 0.04	0.19 ± 0.02	0.19 ± 0.02	0.19 ± 0.03	0.18 ± 0.03
		3	0.20 ± 0.03	0.19 ± 0.03	0.18 ± 0.01	0.18 ± 0.02	0.16 ± 0.02
	Va	0	2.03± 0.10^{a}	2.02 ± 0.27^{a}	2.03 ± 0.25^{a}	2.04 ± 0.15^{a}	2.01 ± 0.09^{a}
		3	0.61 ± 0.04^{b}	0.61 ± 0.09^{b}	0.61 ± 0.08^{b}	0.61 ± 0.08^{b}	0.62 ± 0.05^{b}
Sunflower oil	Car	0	0.19 ± 0.02^{a}	0.18 ± 0.03^{a}	0.18 ± 0.02^{a}	0.20 ± 0.01^{a}	0.19 ± 0.02^{a}
		4	0.06 ± 0.01^{b}	0.05 ± 0.00^{b}	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}
	Eu	0	0.59 ± 0.08^{a}	0.58 ± 0.09^{a}	0.55 ± 0.06^{a}	0.56 ± 0.09^{a}	0.55 ± 0.06^{a}
		4	0.45 ± 0.06^{b}	0.44 ± 0.06^{b}	0.46 ± 0.08^{b}	0.48 ± 0.04^{b}	0.46 ± 0.05^{b}
	Ger	0	0.79 ± 0.06	0.78 ± 0.01	0.79 ± 0.09	0.79 ± 0.09	0.79 ± 0.10
		4	0.81 ± 0.08	0.80 ± 0.04	0.77 ± 0.09	0.78 ± 0.05	0.78 ± 0.08
	Thy	0	0.21 ± 0.04^{a}	0.19 ± 0.02^{a}	0.19 ± 0.02^{a}	0.19 ± 0.03^{a}	0.18 ± 0.03^{a}
		4	0.04 ± 0.01^{b}	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.04 ± 0.00^{b}	0.03 ± 0.00^{b}
	Va	0	2.03± 0.10^{a}	2.02 ± 0.27^{a}	2.03 ± 0.25	2.04 ± 0.15	2.01 ± 0.09
		4	1.95 ± 0.12^{b}	1.96 ± 0.14^{b}	1.94 ± 0.13	1.94 ± 0.19	1.93 ± 0.14

Different small letters in the same row denote statistically significant differences in effective EOCs concentration between food component concentrations. Significance levels: p<0.001.

Table 1. Continuation

Food constituent	EOC	g/100 mL food constituent	Incubation Time (h)				
			0	1	4	8	24
	Car	0	0.19 ± 0.02	0.18 ± 0.03	0.18 ± 0.02	0.20 ± 0.01	0.19 ± 0.02
		4.5	0.18 ± 0.03	0.18 ± 0.04	0.16 ± 0.04	0.17 ± 0.03	0.18 ± 0.03
	Eu	0	0.59 ± 0.08	0.58 ± 0.09	0.55 ± 0.06	0.56 ± 0.09	0.56 ± 0.06
		4.5	0.58± 0.08	0.56± 0.06	0.55± 0.07	0.57± 0.06	0.55 ± 0.06
D-lactose	Ger	0	0.79 ± 0.06	0.78 ± 0.01	0.79 ± 0.09	0.79 ± 0.09	0.79 ± 0.10
		4.5	0.78 ± 0.05	0.79 ± 0.04	0.79 ± 0.08	0.77 ± 0.09	0.77 ± 0.05
	Thy	0	0.21 ± 0.04	0.19 ± 0.02	0.19 ± 0.02	0.19 ± 0.03	0.18 ± 0.03
		4.5	0.19 ± 0.03	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.17 ± 0.03
	Va	0	2.03 ± 0.10	2.02 ± 0.27	2.03 ± 0.25	2.04 ± 0.15	2.01 ± 0.09
		4.5	1.95 ± 0.15	1.93 ± 0.20	1.98 ± 0.15	1.94 ± 0.20	1.94 ± 0.16

3.5 Antimicrobial activity of EOCs at a certain free concentration

Having experimentally demonstrated an interaction between some of the investigated EOCs and the matrix components, which significantly lowered the antimicrobial concentration, we paid attention to determine whether this reduction was responsible for the inhibition of their bactericidal efficacy. For this purpose, the antimicrobial activity of every EOC at the lowest concentration found after exposure with some food component (see details in Section 3.4) was determined. The concentrations of 0.04 mg/mL of Car, 0.46 mg/mL of Eu, 0.65 mg/mL of Ger, 0.03 mg/mL of Thy and 0.62 mg/mL of Va were employed, which corresponded to 20%, 77%, 81%, 15% and 32% of the MIC values for Car, Eu, Ger, Thy and Va, respectively. At these concentrations, the bactericidal capacity of Car, Thy and Va was very poor (< 1 $\log_{10}$ of reduction), while Eu and Ger were able to inhibit bacteria growth after 8 and 1 h of incubation, respectively (**Figure 7**). These outcomes highlight that, of the mechanisms of EOCs antimicrobial activity inhibition after an interaction with food components, the reduction in the EOCs effective concentration available to directly interact with bacteria is one of them.

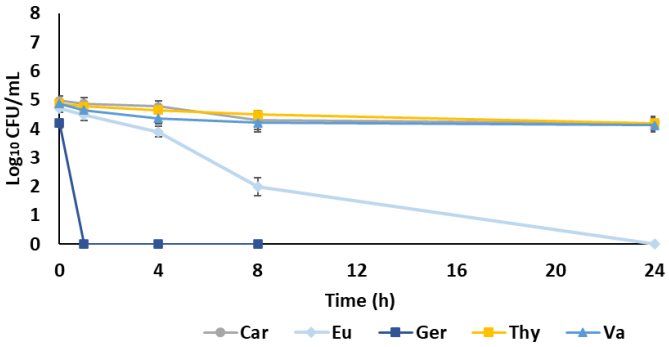

Figure 7. Time-kill curves for *E. coli* treated with carvacrol (Car) (0.04 mg/mL), eugenol (Eu) (0.46 mg/mL), geraniol (Ger) (0.65 mg/mL), thymol (Thy) (0.03 mg/mL) and vanillin (Va) (0.62 mg/mL).

4. Conclusions

In this work, the impact of major food components on antimicrobial efficiency against *E. coli* of five EOCs was investigated. The results of the time-killing curves, which monitor bacterial growth and death over a range of antimicrobial concentrations, revealed that BSA, sunflower oil and some carbohydrates inhibit EOCs antimicrobial activity. These food components either decrease the number of achieved logarithmic reductions to a lesser extent or delay the bacterial killing time. Furthermore, the HPLC quantification of the effective concentration of the five assayed antimicrobials after exposure to BSA, sunflower oil and D-lactose denoted that loss of the free antimicrobial concentration, produced by different types of interactions between EOCs and food components, appeared to be one of the main reasons to explain the inhibition of EOCs antimicrobial activity.

Therefore, beyond the need to investigate the impact of an antibacterial agent from natural sources (EOCs) on the proliferation of a specific bacterial strain for food shelf-life extension purposes, this study underscores the significance of knowing the impact of the interaction of these compounds with diverse food components. This understanding is crucial for selecting the most appropriate agent to effectively minimise any potential microbial contamination in a given food and emphasises the importance of following a holistic approach to optimise food preservation.

In this context, future works should evaluate a wider range of antimicrobial families, together with different protein, lipids and carbohydrates and bacteria strains, to obtain a complete catalogue of antimicrobials-food components-strain interactions. This information is essential for confidently recommending the application of a particular antimicrobial depending on the specific food components present in the food matrix.

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4.2. Major food constituents influence the antibacterial activity of vanillin immobilized onto silicon microparticles against *Escherichia coli*

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Abstract

Antimicrobial filtration materials based on essential oil components (EOCs) immobilized onto silicon oxide particles have been found effective for nonthermal stabilization of liquid foods (i.e., beer, juice, wine, and etc.). However, their antimicrobial efficiency depends on the food matrix. The present work aimed to assess the effect of the major constituents of liquid food matrices on the antimicrobial activity of vanillin immobilized onto silica microparticles. Silicon oxide particles were functionalized with vanillin and characterized. The maximum tolerated concentrations of different food major constituents (i.e., proteins, lipids, carbohydrates, organic acids, alcohols and minerals) were determined against *Escherichia coli* K12. The results showed that organic acid and alcohol had synergetic effects on vanillin-functionalized particles, and the addition of proteins, lipids or some carbohydrates inhibited their antimicrobial activity. No effects on microbial counts were found for mineral salts. The dual combinations between the synergistic and nonsynergistic food constituents showed improved antimicrobial activity compared to single compounds. The data confirmed previous in vitro experiments and could be used to predict the antimicrobial activity of the filtration system when treating real liquid food matrices.

Keywords: interaction; *Escherichia coli*; functionalization; essential oil components; covalent bond

1. Introduction

Sorbates and benzoates in fruit juice (Walker & Phillips, 2018), and sulfites in wines (Ruiz-Rico, García-Ríos, Barat & Guillamón, 2021b), have been typically employed to enhance food safety. However, there are concerns for using these products because their consumption may potentially lead to health problems (i.e., asthma, urticaria, allergic reactions, etc.).

Antimicrobials obtained from natural sources are an emerging alternative as they are effective against a diverse range of microorganisms. Of all natural antimicrobials, essential oil components (EOCs), purified compounds extracted from plants are generally recognized as safe (GRAS) by the US Food and Drug Administration (Falleh et al., 2020; Mukurumbira et al., 2022).

Despite demonstrated effectiveness of EOCs as antimicrobial compounds (Chouhan et al., 2017), their direct use is limited due to their intrinsic properties, such as poor solubility in aqueous systems, high volatility and strong flavor (Ruiz-Rico et al., 2017). To overcome these limitations, immobilization by covalently anchoring the active biomolecule onto different food grade surfaces has been recently proposed as an eco-friendly method to preserve foods and drinks (Gómez-Llorente et al., 2023; Ruiz-Rico & Barat, 2021a). Ruiz-Rico et al. (2017) first reported the immobilization of carvacrol, eugenol, thymol and vanillin onto different silica support, indicating that the EOC anchoring strategy not only produced greater stability, but also better antimicrobial activity and a longer shelf life.

Of all the possible applications of EOCs-functionalized materials, their use as filtration elements to achieve the cold pasteurization of liquid food has been extensively studied. Peña-Gómez et al. (2019a) tested the microbial quality of water inoculated with *E. coli* K12 after being filtered through carvacrol-, eugenol-, thymol- and vanillin-functionalized amorphous silica particles. In their study, most of the designed antimicrobial devices achieved total *E. coli* inactivation and reached the WHO-compliant levels for the drinking water category (WHO, 2022). Functionalized silica supports also maintained antibacterial activity after washing and filtering different volumes of inoculated water. Further studies have focused

on evaluating the effectiveness of the proposed antimicrobial filtration system on more complex food matrixes. Peña-Gómez et al. (2019b) filtered apple juice inoculated with *E. coli* through eugenol- and vanillin-functionalized silica particles. These authors indicated a significant role in the elimination of both *E. coli* and juice microbiota (mesophilic, psychrophilic and mold/yeast) for the first 120 refrigerated storage days. Peña-Gómez et al. (2020) also examined the properties of immobilized carvacrol, eugenol, thymol and vanillin on silica particles to reduce *E. coli* in craft beer inoculated with this microorganism. They found that all the EOCs were able to drastically reduce microorganisms, even after filtering a high volume of inoculated craft beer. The same study also examined the possible sensory changes in the beer that resulted after passage through EOCs-functionalized particles and no effect was detected. Likewise, Ruiz-Rico et al. (2021b) analyzed the capacity of immobilized eugenol and vanillin for reducing the typical microorganisms present in wines (*A. aceti, L. plantarum, D. bruxellensis, S. cerevisiae* and *Z. bailii*). The study indicated similar antimicrobial levels to those reported after pulsed electric fields or high hydrostatic pressure treatments. Despite the good results obtained in water, juice, beer or wine, the use of this methodology in other matrices, such as milk or tigernut-derived beverages, is ineffective for lowering the microbial load (Garrido-Momparler, 2021; Roselló-Tomás, 2019).

Considering all these results, evidently the antimicrobial activity of particles is conditioned by the food matrix in which they are applied. Therefore, the objective of this work was to determine the influence of different constituents present in liquid food matrices (proteins, lipids, carbohydrates, organic acids, alcohols, minerals) on the antimicrobial activity of vanillin immobilized onto silica microparticles. As most of the above-mentioned studies in real food matrices have evaluated the antimicrobial activity of vanillin immobilized onto silicon oxide supports against *Escherichia coli* K12 bacteria, the same bacteria and strain were herein selected to establish direct correlations between the inhibition of vanillin antimicrobial activity in not only a certain real product, but also in the presence of a certain food constituent.

2. Materials and methods

2.1. Chemicals

Vanillin, (3-Aminopropyl) triethoxysilane (APTES), sodium borohydride, bovine serum albumin, pectin and silica particles (50-110 µm) were purchased from Sigma-Aldrich (Madrid, Spain). Methanol, ethanol, Bovine Serum Albumin (BSA), D-sucrose, D-lactose, citric acid, NaCl, KCl, Na_2HPO4, KH_2PO_4, HCl, $MgCl_2$, $CaCl_2$, $NaHCO_3$ and microbiological media were provided by Scharlab (Barcelona, Spain). Sunflower oil was purchased in a local supermarket.

2.2. Synthesis of the vanillin-functionalized silica particles

Functionalized silica particles were prepared by the covalent immobilization of vanillin onto a bare silica support (**SiO_2**) following the surface silanization procedure described by Ruiz-Rico et al. (2017). For this purpose, 40 g of the silica particles (50-110 µm) were reacted with 14 mL of APTES in the presence of isopropanol (200 mL) for 1 h while stirring (20 g) at room temperature (RT). Afterward, 8 g of vanillin dissolved in 120 mL isopropanol were added. The mixture was left for 1 h to stir at RT. The obtained solid was separated by centrifugation (9500 *g*, 8 min) and mixed with 1.2 g of sodium borohydride in the presence of methanol (500 mL) overnight to reduce the formed imine bond to an amine one. Reduced vanillin-functionalized silica particles were separated by centrifugation (9500 *g*, 8 min) and washed with distilled water (pH 4) until no vanillin leaching was observed. The vanillin leaching analysis was done by high-performance liquid chromatography (HPLC) according to the procedure described by Pérez-Esteve et al. (2016). After confirming that there was no vanillin leaching from particles, the obtained solid was left to dry at 30°C for 24 h, which yielded the **SiO_2-Va** material.

2.3 Materials characterization

SiO_2 and **SiO_2-Va** were characterized by standard techniques. The morphological analysis of particles was performed by field emission scanning electron microscopy (FESEM). The FESEM images were acquired by a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. Elemental

analyses were performed for C, H and N in a CHNOS Vario EL III model (GMHB, Germany). The degree of functionalization (α) was determined by elemental analyses and expressed as mg vanillin/g SiO_2 (Ruiz-Rico et al., 2017).

2.4 Antimicrobial analysis

2.4.1 Bacteria culture

Escherichia coli K12 (CECT 433, Colección Española de Cultivos Tipo, Valencia, Spain), maintained in cryovials, was used following the CECT instructions. To this end, Plate Count Agar (PCA) and Tryptone Soy Broth (TSB) were used to grow the microorganism. Then the bacterial stock was stored at 4 °C on PCA. To obtain an inoculum with an approximate microbial density of 10^8 CFU/mL, the cells from a colony were placed inside a test tube with 10 mL of TSB to be incubated at 37 °C overnight.

2.4.2 Preparing food constituents

The different food constituents used in this work were chosen after taking the typical composition of liquid foods (i.e., beer, juice, wine or milk) as a reference. For this purpose, the presented natural food constituents and the concentration ranges were selected based on specific international legislation and standards, food composition databases and scientific research analyses (British Standards Institute, 2019; Carrin et al., 2004; Edelmannet al., 2003; European Parliament and Council, 2013; Gill & Donaghy, 2004; He et al., 2007; Mitchell Jr et al., 2014; Sakamoto & Konings, 2003; USDA, 2023; Wang et al., 2020). To prepare the protein food constituent, 0.005-0.03 g bovine serum albumin/100 mL was used. For lipids, 0.5-5 g sunflower oil/100 mL was selected. With carbohydrates, three different food constituents were prepared by adding 0.5-5 g D-lactose/100 mL, 2-12 g D-sucrose/100 mL and 0.001-0.005 g pectin/100 mL. For the acid and alcohol conditions, 0.05-0.2 g citric acid/100 mL and 2-12 g ethyl alcohol/100 mL were employed, respectively. To evaluate the effect of minerals, the hard water model proposed by the EN 1276 standard of the British Standards Institute (British Standards Institute, 2019) was applied. In particular, two different solutions (A and B) were prepared. For solution A, 19.82 g of $MgCl_2$ and 46.24 g $CaCl_2$ were dissolved

in 1000 mL of distilled water and were sterilized in an autoclave. With solution B, 35.02 g of $NaHCO_3$ were dissolved in 1000 mL in another flask and sterilized using a membrane with a 0.45-µm pore size. Then 6 mL of solution A and 8 mL of solution B were mixed, and the sterilized distilled water was made up to a final volume of 1000 mL. Finally, the solution was adjusted to pH 7 ± 0.2 using 1 M NaOH or HCl. This solution was considered 1-fold water hardness. Solutions up to 50-fold water hardness were prepared by mixing solutions A and B with aqueous media in the proper proportions.

All the food constituents were prepared in distilled water and sterilized by filtration through sterilized membrane filters (0.45-µm pore size; Millipore, Merck, Italy). After preparing the food constituents, they were inoculated with a bacterial density of 3 $\log_{10}$ CFU/mL. For this inoculation, serial dilutions from the inoculum were done. For calculations, the cell concentration of the inoculum was assessed by measuring the optical density at 600 nm using a Helios Zeta UV-VIS instrument (Thermo Scientific, Hampton, New Hampshire, USA). This concentration was corroborated by determining the *E. coli* concentration ($\log_{10}$ CFU/mL) in the inoculated food constituents by following the plate count technique (see Section 2.4.4).

2.4.3 Filtration procedure

In order to treat the inoculated food media with **SiO_2-Va**, the filtration procedure described by the International Standard Organization (ISO) (ISO 9308-1, 2014) was employed with minor modifications. A stainless-steel manifold filtration system (Microfil®, Merck Millipore, Darmstadt, Germany), connected to an Erlenmeyer flask to collect the sample, was used. Three layers of filtration materials were placed on the manifold. From top to bottom, they were: "2 cm thickness of **SiO_2-Va** (25 g) (ca. 26 cm^3 of the filtration material volume)"; "cellulosic paper"; "a cellulose membrane" (47 mm in diameter, 0.45-µm pore size; Millipore, Merck).

2.4.4 Antimicrobial activity quantification

All the microbial analyses were carried out by seeding the cellulose membrane on the selective medium Tryptone Bile X-Glucuronide agar (TBX) (Scharlab, Barcelona, Spain). Plates were incubated at 37 °C for 24 h. Three microbiological replicates were carried out in all the assays. A control (inoculated water filtered through **SiO_2**) was included in the assay. The results were expressed as logarithmic reduction values (LRV), calculated by **Equation 1** (Peña-Gómez et al., 2019a):

$$LRV = log_{10}\, CFU/100mL\,(\boldsymbol{SiO_2}) - log_{10}\, CFU/100mL\,(\boldsymbol{SiO_2\text{-}Va}) \quad \text{(Equation 1)}$$

The antimicrobial effect of the **SiO_2-Va** particles was considered null when LRV was ≤ 2 $\log_{10}$ CFU/100 mL.

2.5 Effect of media on immobilized vanillin antimicrobial activity

In order to quantify the effect of the different food constituents on the antimicrobial activity of **SiO_2-Va** as a filtering system, two different experiments were conducted. First, to determine the maximum tolerated concentration (MTC), defined as the maximum concentration of each food constituent that allowed LRV ≥ 2 $\log_{10}$ CFU/100 mL to be continued, 100 mL of each inoculated food model were filtered (Test 1). The distilled water inoculated with *E. coli* was used as a control to evaluate **SiO_2-Va** antimicrobial activity in the absence of food constituents.

After determining this value and taking into account that **SiO_2-Va** should reduce to at least 2 $\log_{10}$ CFU/100 mL, either the maximum tolerated volume (MTV) or the maximum volume of the food constituent that could be filtered was quantified (Test 2). For this purpose, increasing volumes of each food constituent were continuously filtered. The concentration of each food constituent medium used in Test 2 was based on the results obtained in Test 1. For the food constituents in which **SiO_2-Va** antimicrobial activity was inhibited, the highest concentration before reaching the MTC was used. For the food constituents that exhibited antimicrobial activity themselves, the maximum concentration of the compound that did not present any bactericidal effect was employed.

Finally, the food constituent effect (FCE) on **SiO_2-Va** antimicrobial efficiency was calculated according to **Equation 2**:

$$FCE\ (\%) = \frac{MTV(w) - MTV(constituent)}{MTV(w)} \times 100 \quad \text{(Equation 2)}$$

where MTV (w) and MTV (constituent) are the MTV for distilled water and for each food constituent, respectively.

The results were interpreted as follows: hindering effect (FCE < 0); noninteractive effect (FCE= 0); synergetic effect (FCE > 0).

The effect of the concentration or volume of each food constituent tested on **SiO_2-Va** antimicrobial activity was graphically represented when **SiO_2-Va** reduced to at least 2 $\log_{10}$ CFU/100 mL of bacteria.

2.6 Statistical analysis

Data were statistically processed by Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). The differences between antimicrobial activity due to distinct food constituent concentrations or differing volumes were determined by a one-way analysis of variance (one-way ANOVA) with a 95% confidence interval (95%CI).

3. RESULTS

3.1 Vanillin support characterization

SiO_2 and **SiO_2-Va** were morphologically characterized by FESEM. As **Figure 1** depicts, the size of **SiO_2** and **SiO_2-Va** ranged between 50 and 110 µm, as specified on the product data sheet. The functionalization process did not affect either the appearance or the structure of supports. Regarding the organic content matter of **SiO_2-Va** (α), the elemental analysis revealed that **SiO_2-Va** contained 40.0 ± 0.1 mg of vanillin / g of silica microparticles. The surface size and degree of the functionalization values agree with previous studies, which employed the same particles and functionalization procedures (Peña-Gómez et al., 2019b; Ribes et al.,

2019; Ruiz-Rico et al., 2017). This indicates that **SiO_2-Va** particles were synthesized in a homologous way to previous works.

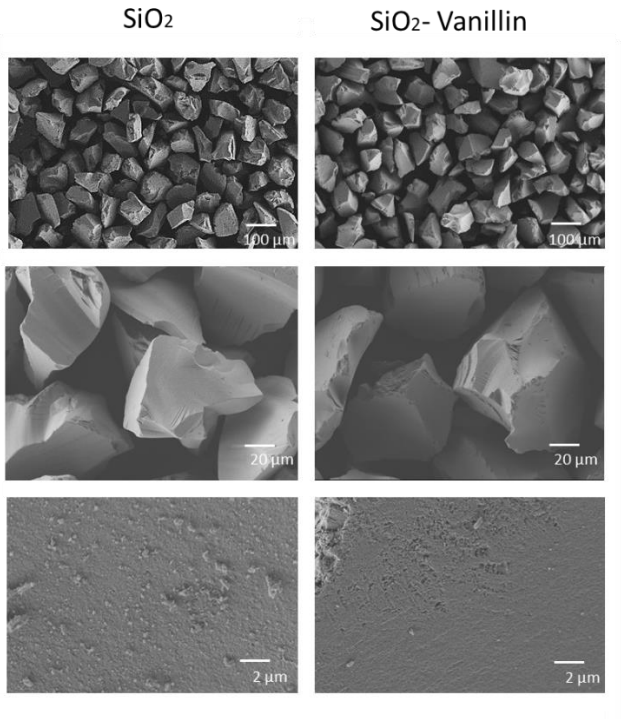

Figure 1. Characterization of particle size and particle shape by FESEM.

3.2 Effect of the food model concentration

After confirming the correct **SiO_2-Va** functionalization, the antimicrobial activity of the proposed filtration system was first assessed by filtration 100 mL of inoculated distilled water at a neutral pH. An LRV of 2.56±0.09 was obtained for **SiO_2-Va** antimicrobial activity. This value agrees with that reported by Peña-Gómez et al. (2019a) when employing the same particles, immobilization methodology and reference microorganism for the microbial treatment of drinking water.

Having confirmed **SiO_2-Va** antimicrobial activity, the MTC of the major food constituents under study was quantified.

3.2.1 Proteins and lipids

For proteins, the MTC was 0.01 g BSA/100 mL (LRV 2.45±0.17) (**Figure 2A**). This protein concentration value was lower than the minimum content normally found in vegetable drinks, such as milk (3 g/100 mL), soy milk (ca. 3.5 g/100 mL) or tigernut-derived beverages (ca. 1.3 g/100 mL) (USDA, 2023).

The strong effect of proteins on reducing **SiO_2-Va** antimicrobial activity agrees with previous works, which evaluated the effect of adding protein to media using different EOCs and microorganisms. In a study in which carvacrol was used to reduce *Staphylococcus aureus*, Juven et al. (1994) observed no reduction in bacteria in 0.9 g BSA / 100 mL media. Likewise, Veldhuizen et al. (2007) indicated that carvacrol antimicrobial activity against *Listeria monocytogenes* was inhibited when adding 1 g BSA/100 mL to media. To clarify the possible mechanisms that hinder the antimicrobial effect of vanillin in the presence of BSA, different studies have been conducted. Guimarães et al. (2019) reported that proteins are able to bind with the phenolic compounds of EOCs by blocking the antimicrobial action of hydroxyl groups. In another study, Huang et al. (2022) specified that Van der Waals and hydrogen bonds are the main bonding forces between amino acids of BSA and the citronellol EOC. As hydroxyl groups are considered to be the main chemical group of EOCs responsible for antimicrobial activity (Ruiz-Rico et al., 2017), interaction with proteins would inhibit their antimicrobial activity.

For lipids**, Figure 2B** shows that their MTC was 3 g of sunflower oil / 100 mL (LRV 2.31±0.12). This value is significantly higher than the MTC for proteins. This concentration also covers a large number of liquid foods, such as juice, wine, beer, and two of the three milk categories: skimmed and semi-skimmed (European Parliament and Council, 2013; USDA, 2023). The obtained results agree with the reported data. Cava et al. (2007) evaluated the antimicrobial activity of cinnamon and clove essential oil against *L. monocytogenes* in semi-skimmed milk media (1.5-1.8 g total lipid/100 mL) and whole milk media (> 3.5 g total lipid/100 mL). Their

study indicated that the increase in lipid content led to the antimicrobial inactivation of EOCs from 3 $\log_{10}$ CFU/mL of bacteria reduction in semi-skimmed media to 1 $\log_{10}$ CFU/mL in whole milk media. Cui et al. (2016) and Mejlholm and Dalgaard (2002) suggested that the presence of lipids in the medium creates a protective layer that surrounds the bacterial membrane, and it prevents any contact between bacteria and the functional groups of the antimicrobial agent. This mechanism could also explain loss of **SiO_2-Va** antimicrobial activity.

3.2.2. Carbohydrates

For lactose, **Figure 2C** shows that the MTC was 3 g lactose/100 mL media (LRV 2.24±0.08). This lactose concentration is lower than those commonly found in whole (4.81 g/100 mL), semi-skimmed (4.89 g/100 mL) and skimmed milk (5.05 g/100 mL) (USDA, 2023). Regarding sucrose, **Figure 2D** indicates that **SiO_2-Va** reduced to at least 2 $\log_{10}$ CFU/mL of bacteria at all the tested sucrose concentrations. However, this reduction significantly decreased ($p<0.05$) when media contained the highest sucrose concentration (12 g sucrose/100 mL) with an LRV of 2.06±0.02. The sucrose concentration covers the most widely consumed fruit-derived beverages, such as apple juice, orange juice, wine, etc. (USDA, 2023). The effect of sucrose on **SiO_2-Va** agrees with the results reported by Tserennadmid et al. (2010), who incorporated 1-8 g sucrose/100 mL into culture media. In their study, the antimicrobial activity of marjoram essential oil was maintained at all the tested concentrations, but was significantly lower for 8 g sucrose/100 mL.

For more complex carbohydrates like pectin (**Figure 2E**), the presence of a higher concentration of 0.001 g pectin/100 mL sufficed to inhibit **SiO_2-Va** antimicrobial activity (LRV 2.62±0.09 for 0.001 g pectin/100 mL). This concentration was lower than the soluble pectin content reported for orange juice (ca. 0.004 g/100 mL) (Bizzani et al., 2020) or wine (ca. 0.01 g/100 mL) (Han & Du, 2022).

3.2.3. Organic acids and alcohols

Regarding organic acids, **SiO_2-Va** were active as an antimicrobial at all the tested concentrations (0.05-0.2 g citric acid/100 mL) with an LRV of 2.45±0.09 for 0.2 g citric acid/100 mL (**Figure 2F**). The different tested concentrations did not

significantly change the bacteria reduction counts (p> 0.05). The highest tested concentration was lower than that present in orange juice (0.7 g/100 mL) (USDA, 2023). However, higher concentrations of 0.2 g citric acid/100 mL could not be tested in this study because bacteria did not grow (data not shown), probably due to media acidification. The reduction in bacteria growth by organic acids could be because of their capacity to penetrate and change bacterial membrane permeability (Chung et al., 2003).

For the alcohol food constituent, **SiO_2-Va** maintained the antimicrobial activity at all the tested concentrations (2-12 g ethyl alcohol/100 mL) with an LRV of 2.40±0.12 for 12 g ethyl alcohol/100 mL (**Figure 2G**). The different food constituent concentrations did not significantly change the bacteria reduction counts (p> 0.05). In this study, the maximum tested concentration was 12 g ethyl alcohol/100 mL because higher concentrations did not result in *E. coli* growth (data not shown). The range of concentrations tested in this work represented the most widely consumed alcoholic beverages, such as beer (4 g/100 mL) or wine (10 g/100 mL) (USDA, 2023).

3.2.4. Mineral salts

For the situation in which inorganic salts were present, **Figure 2H** shows how **SiO_2-Va** was active, even up to concentrations that were 50-fold higher than what is considered hard water (British Standards Institute, 2019) (LRV 2.68±0.01 at 50-fold higher). In addition, increasing the values of minerals did not statistically modify (p> 0.05) **SiO_2-Va** antimicrobial activity. These results agree with those reported by Oussalah et al. (2007), who tested the antimicrobial activity of EOCs on pretreated alginate films with different $CaCl_2$ contents.

3.3 Effect of the food model volume

After quantifying the effect of the MTC for each food model, different experiments were carried out to calculate the MTV of the different food constituents either individually (**Figure 3**) or combined (**Figure 4**). From these data, the FCE, or the degree of synergy or hindering, in a given food constituent medium was also calculated (**Figure 5**).

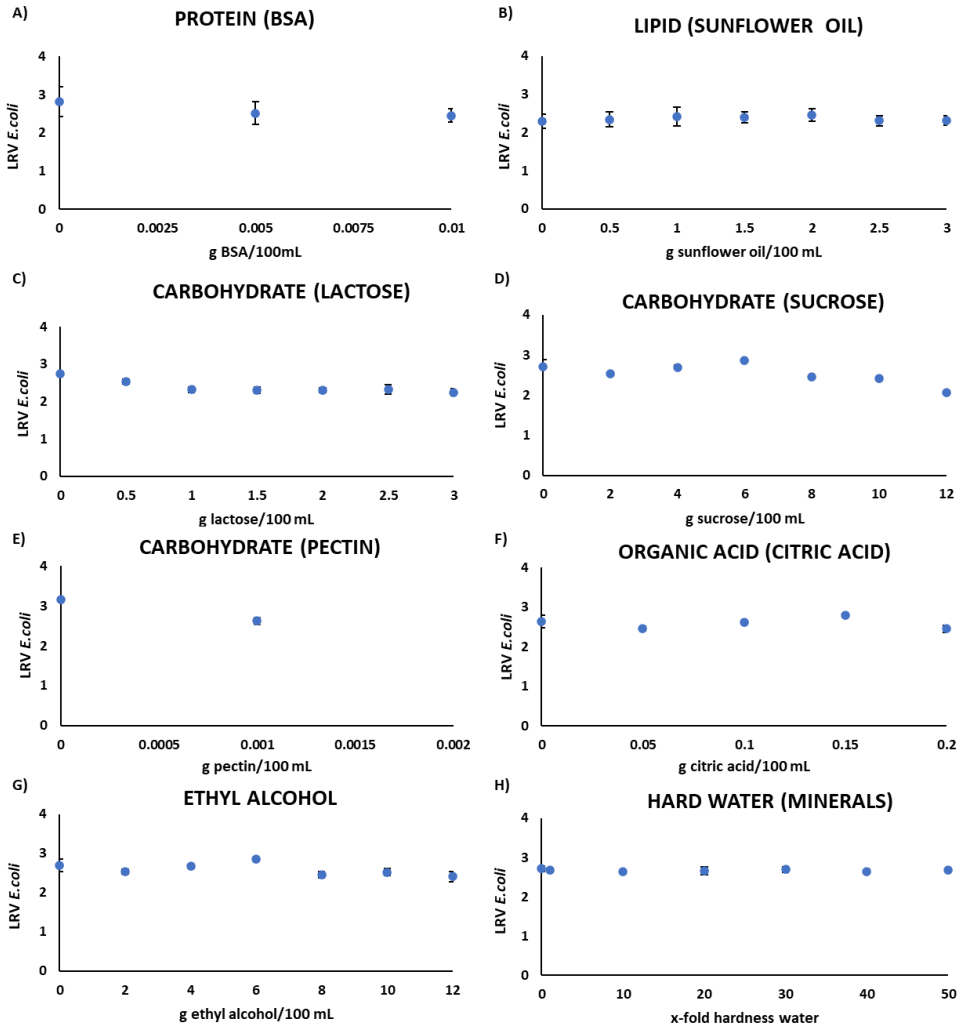

Figure 2: Effect of the different proportions of (A) bovine serum albumin (BSA); (B) sunflower oil; (C) lactose; (D) sucrose; (E) pectin; (F) citric acid; (G) ethyl alcohol and (H) mineral salts dissolved in distilled water on **SiO_2-Va** antimicrobial activity against *E. coli*. The results are expressed as LRV (logarithmic reduction value) ($\log_{10}$ CFU/100mL). Mean value±SD (n=3).

3.3.1 Simple matrices

For distilled water**, Figure 3A** shows that 1300 mL were the maximum volume filtered through **SiO_2-Va** without them losing their condition as an active material. In this case, loss of antimicrobial activity might be because successive filtrations would cause organic matter to accumulate in the filter, which would result in less contact between bacteria and the **SiO_2-Va** active groups.

In relation to the media containing protein (0.01 g BSA/100 mL), **Figure 3B** shows that the MTV was only 100 mL of solution. Consequently, the FCE was -92%, which indicates a marked hindering effect on the functionalized particles when proteins were added to media. For lipids (**Figure 3C**), at the 3 g sunflower oil/100 mL concentration, the filter was active until 300 mL of solution (MTV), reaching a FCE value of -77%.

For carbohydrates, **SiO_2-Va** was active as an antimicrobial until the filtration of 300 mL of solution with 3 g lactose/100 mL (LRV 2.29±0.08), 1300 mL for 12 g sucrose/100 mL (LRV 2.33±0.18) and 200 mL for 0.001 g pectin/100 mL (LRV 2.33±0.01) **(Figures 3D**, **3E** and **3F**). According to the filtered volume, the FCE (represented in **Figure 5**) was -77%, 0% and -84% for the lactose, sucrose and pectin food constituents, respectively. This finding indicates remarkable hindering effects for lactose and pectin, but no interactive effects for sucrose.

On the presence of acid (0.2 g citric acid/100 mL) and alcohol (12 g ethyl alcohol/100 mL) in media, **Figures 3G** and **3H** show how **SiO_2-Va** maintained antimicrobial activity throughout the assay. That is, **SiO_2-Va** showed > 2 $\log_{10}$ CFU/100mL of bacteria reduction after filtering 2500 mL with an FCE>100 %. This scenario indicates that these food constituents provoke a synergetic effect on **SiO_2-Va** antimicrobial activity compared to the distilled water results. The LRV was 2.43±0.11 and 2.29±0.12 for citric acid and ethyl alcohol, respectively.

After adding inorganic compounds, the hard water volume effect was also studied (**Figure 3I**). Antimicrobial activity (LRV 2.02± 0.02) was recorded in the same volume as the control (1300 mL, FCE of 0 %), which indicates that no interactive effects derive from adding minerals to media.

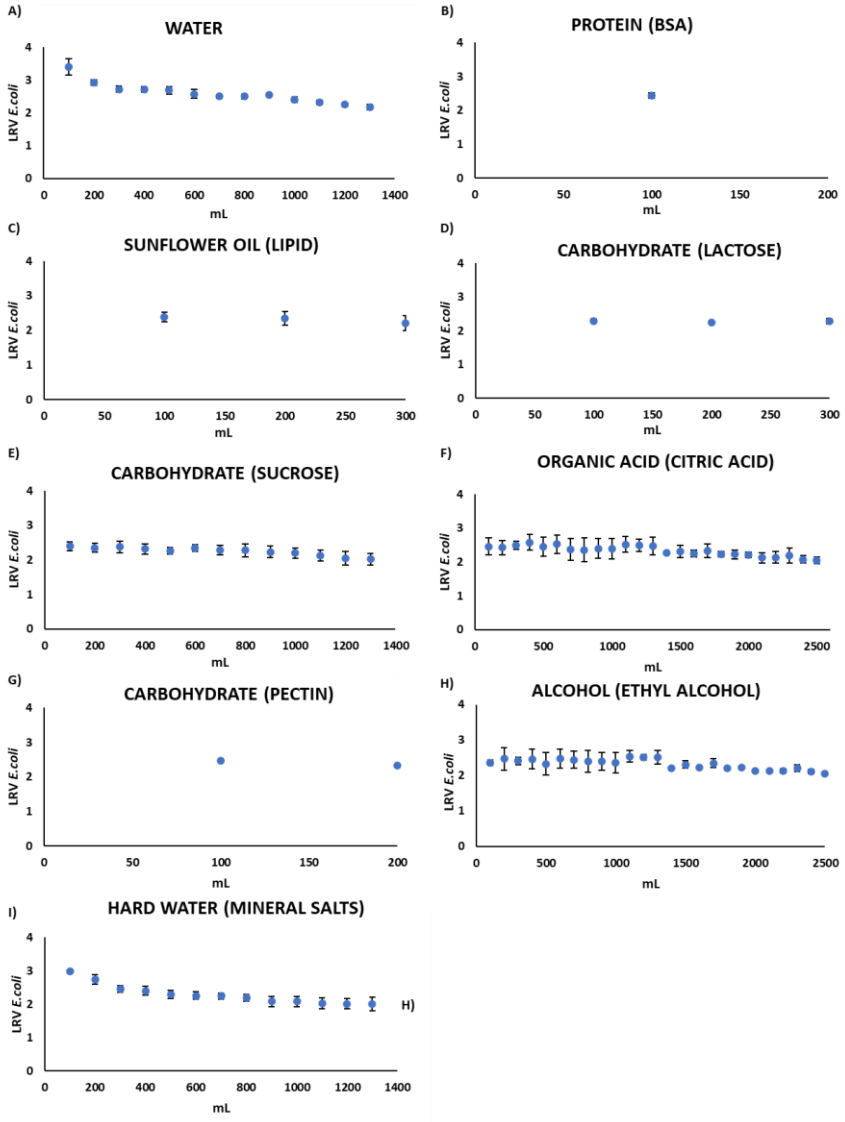

Figure 3. Effect of the volume of the food constituents filtered upon **SiO_2-Va** antimicrobial filtration activity: (A) distilled water, (B) 0.01 g BSA/100 mL, (C) 3 g sunflower oil/100 mL (D), 3 g lactose/100 mL, (E) 12 g sucrose/100 mL, (F) 0.001 g pectin/100 mL, (G) 0.2 g citric acid/100 mL, (H) 12 g ethyl alcohol/100 mL and I) 1-fold hardness of water. The results are expressed as LRV (log_{10} CFU/100 mL). Mean value±SD (n=3).

3.3.2. Dual matrices

After evaluating the effect of the single food constituents with **SiO_2-Va** on the MTV, the effect of the dual combinations of food constituents was evaluated to mimic real foods in which different constituents are found in the same food matrix. The following dual combinations were tested by reproducing the composition of sweet wine, apple juice and orange juice (USDA, 2023), respectively: 6 g sucrose/100 mL and 12 g ethyl alcohol/100 mL; 6 g sucrose/100 mL and 0.2 g citric acid/100 mL; 0.001 g pectin/100 mL and 0.2 g citric acid/100 mL.

Figure 4 shows the LRV of *E. coli* K12 (log_{10} CFU/100 mL) after filtering increasing volumes of the dual matrices. As observed, the combinations of sucrose and ethyl alcohol (**Figure 4A**), and of sucrose and citric acid (**Figure 4B**), increased ($p<0.05$) the MTV of the dual solutions compared to the single sucrose food model (1300 mL), which allowed the maximum tested volume (2500 mL) to be filtered. When comparing **SiO_2-Va** antimicrobial activity in ethyl alcohol (LRV 2.39±0.06) and citric acid (LRV 2.43±0.11) individually to the dual mixtures, the LRV slightly increased. With the pectin and citric acid mixture (**Figure 4C**), the MTV increased from 100 mL (single pectin) to 600 mL (LRV 2.25±0.01).

These results suggest that the combination between a hindering or nonsynergetic food constituent with synergetic compounds increases global antimicrobial activity. Nevertheless when the food constituent had a significant hindering effect, such as pectin (FCE of -84 %), the mixture with the synergetic food constituent failed to obtain the FCE with positive values (FCE of -54 %).

These results would explain the different antimicrobial behaviors of the vanillin-functionalized silicon oxide particles when used to microbiologically stabilize different liquid foods. Having employed apple juice as a food matrix, Peña-Gómez et al. (2019b) reported an *E. coli* reduction of ca. 3 log_{10} CFU/mL after filtering this matrix through vanillin-functionalized particles. According to the international food database, apple juice contains 0.09, 0.29, 1.39 and < 0.25 g/100 mL of protein, lipids, sucrose and lactose, respectively. It also has a low pH and does not present pectin due to the enzymatic action of pectinases (Carrin et al., 2004; He et al., 2007; USDA, 2023). Considering this composition, and in light of the results herein

obtained, the efficiency of **SiO_2-Va** in these media would be explained by the fact that all the contents of food constituents were below the MTC. Furthermore, juice has an acid pH, which is a synergistic factor.

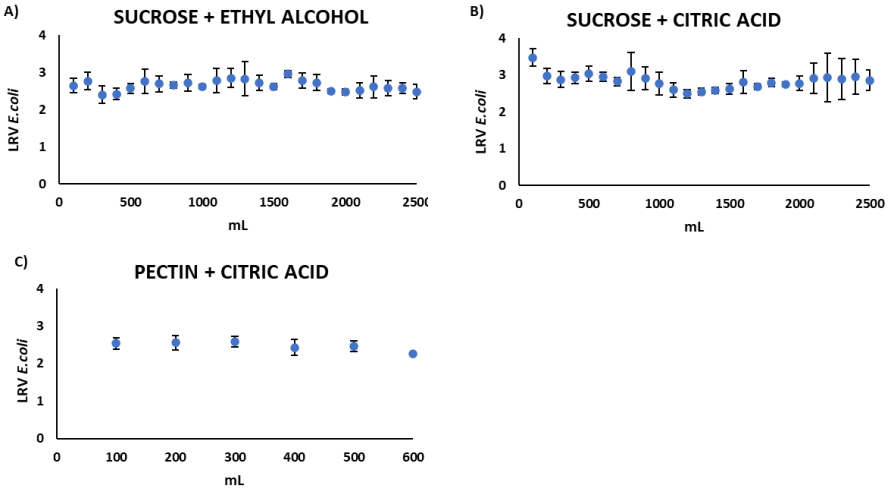

Figure 4. Effect of the volume of the food constituents filtered upon **SiO_2-Va** antimicrobial filtration activity: (A) 6 g sucrose/100 mL and 12 g ethyl alcohol/100 mL, (B) 6 g sucrose/100 mL and 0.2 g citric acid/100 mL and (C) 0.2 g citric acid/100 mL and 0.001 g pectin/100 mL. The results are expressed as LRV (log_{10} CFU/100 mL). Mean value±SD (n=3).

Regarding beer, Peña-Gómez et al. (2020) also demonstrated the antimicrobial effect of SiO_2 particles functionalized with vanillin on *E. coli*. According to the international food database, protein composition is 0.45 g/100 mL, but does not contain lipids, sucrose or lactose. It records a low pH and ethyl alcohol of 3.9 g/100 mL (USDA, 2023). In this case, although protein content was higher than the MTC, the absence of lipids and carbohydrates, together with the low pH and notable alcohol content, would explain the observed effectiveness of **SiO_2-Va**.

The efficiency of the SiO_2 functionalized with vanillin to reduce the quantity of *Acetobacter aceti* in white wine as reported by Ruiz-Rico et al. (2021b) can be, once again, explained by the matrix composition. This product contains protein, lipid, total sugars and ethyl alcohol of 0.07, 0, 0.96 and 10.3 g/100 mL, respectively

(USDA, 2023). Although the protein composition of white wine exceeds the MTC reported in this study, the other food constituents would help to maintain the antimicrobial activity herein indicated.

Likewise, lack of bacterial reduction, as reported by Garrido-Momparler (2021) or Roselló-Tomás (2019) when respectively filtering milk or tigernut-derived beverages through functionalized vanillin particles, could be explained by the marked effect of the main constituents of its matrix on inhibiting **SiO_2-Va** antimicrobial activity. For milk and tigernut-derived beverages, the proteins, lipids and total sugars contents are 3.36 and 1.27 g/100 mL, 1.9 and 2.53 g/100 mL and 4.89 and 4.22 g/100 mL, respectively (USDA, 2023), which are all higher than the reported MTC.

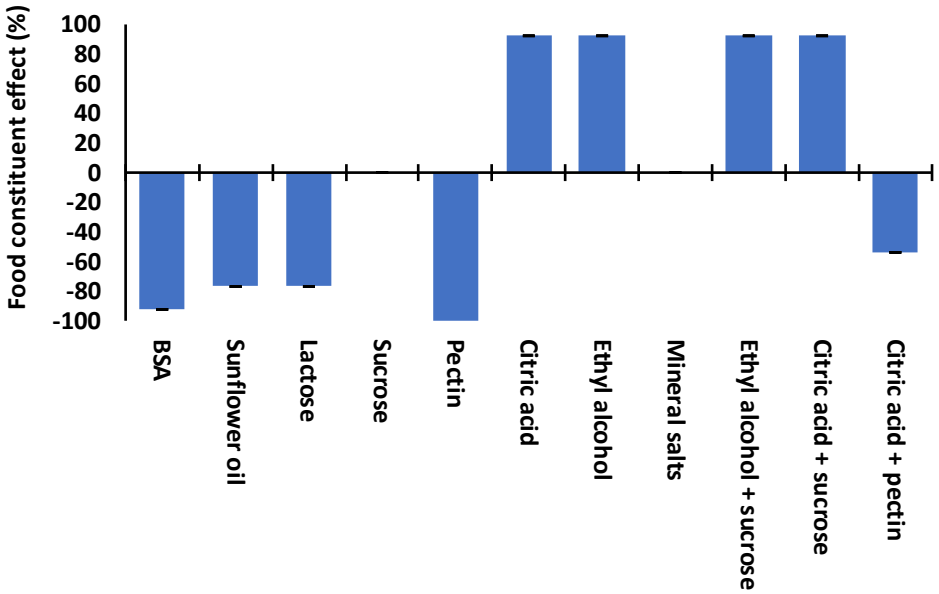

Figure 5. Food constituent effect (FCE) on **SiO_2-Va** antimicrobial efficiency. The results are expressed as percentage (%). Mean value ± SD (n = 3).

4. Conclusions

The antimicrobial activity of vanillin immobilized onto SiO_2 particles is clearly conditioned by the constituents (type and concentration) present in the food matrix. The presence of proteins, lipids and carbohydrates in media hindered **SiO_2-Va** antimicrobial activity, while organic acid and alcohol compounds improved it. Besides, the presence of minerals in media did not change antimicrobial activity. These results explain the diversity in antimicrobial activity found in previous studies when using juice, beer, wine, vegetable beverages, etc., and sets a starting point to predict this antimicrobial system's capability to improve the microbial quality of a concrete liquid food type based on its composition. Moreover, after verifying the importance of the food matrix composition on the antimicrobial activity of immobilized vanillin, further studies should be carried out to evaluate the effect of the matrix on the antimicrobial activity of other bioactive compounds of different natures (terpene, organic acid, fatty acids, etc.) and against other microorganisms like gram-positive bacteria. These data would allow the most effective antimicrobial to be selected by considering both the minimum inhibitory concentration of a certain bacterium and the concentration of a certain constituent in the food matrix.

Acknowledgments

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5. CHAPTER II

Effect of immobilized EOCs against spoilage bacteria

5.1. *Non thermal inactivation of Alicyclobacillus acidoterrestris and guaiacol production in orange juice by using silica microparticles functionalized 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-flavor that leads to 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. In this work, a non-thermal preservation technology based on silica microparticles functionalized with essential oil components was applied to orange juice inoculated with five different *A. acidoterrestris* strains to control bacterial growth and guaiacol production. The immobilized essential oil components were applied following two different approaches: contact with juice in uniform dispersion; filtering juice through a layer of functionalized microparticles. First, the potential of the different pure essential oil components was evaluated by *in vitro* experiments in culture medium. These tests revealed that eugenol and thymol were the most effective antimicrobials, while vanillin and geraniol displayed moderate or minimal effectiveness, respectively. According to these results, both the free and immobilized forms of eugenol and thymol were incorporated into orange juice inoculated with *A. acidoterrestris*, with good antimicrobial properties against all the tested strains. The juice incubation with MIC×0.5 of immobilized eugenol and thymol for 24 h reduced guaiacol production to undetectable levels. The inoculated juices were also filtered through eugenol and thymol particles. This treatment was able to inhibit guaiacol production by the microorganism. These findings highlight the potential of harnessing the antimicrobial properties of immobilized essential oil components to mitigate *A. acidoterrestris* contamination risks during juice production. By these approaches, juice producers could meet consumer demands for safe, premium-quality products, while ensuring extended shelf life and minimizing flavor defects and, thus, food waste.

Keywords: spoilage microorganism, eugenol, thymol, alternative preservation methods

1. Introduction

The food industry is currently facing several challenges, especially in quality preservation, microbiological safety and food waste reduction areas. 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 in both industry and research, the Food and Agriculture Organization of the United Nations (FAO) reports staggering figures, with estimates showing that around 1.3 billion tons of agricultural products are annually wasted (FAO, 2019). In addition, Mudaliar et al. (2023) highlight a significant problem in 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 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 a significant concern (Kaur et al., 2023; 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' color or texture nor produces observable gases that can cause package swelling (Sinigaglia et al., 2003). However, *A. acidoterrestris* bacterium is able to metabolize certain compounds present in juices, which results in undesirable flavors that render the product unsuitable for consumption. Of all the metabolites produced by *A. acidoterrestris*, guaiacol is widely recognized as a key compound involved in quality loss by conferring contaminated products a smoky or medicinal off-flavor. 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 **Figure 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.

If all these facts are taken into account, controlling *A. acidoterrestris* growth and minimizing guaiacol production, which are recognized key indicators of *A. acidoterrestris* activity in orange juice, are critical for preserving the sensory properties and consumer attractiveness of juice products, and fall in line with the priority of maintaining product quality (Pérez-Cacho et al., 2011).

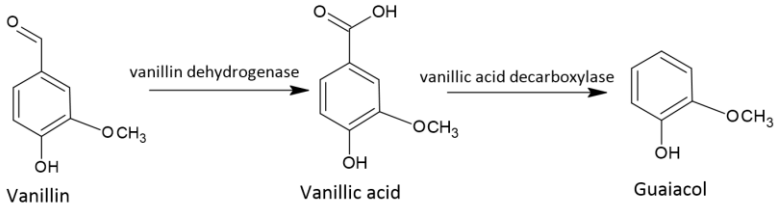

Figure 1. Synthesis of guaiacol by *A. acidoterrestris* (Cai et al.,2015).

Many approaches have been described to control *Alicyclobacillus* spp. contamination, but lots of them are based on heat treatments and are ineffective because it is a highly thermoresistant bacterium (Wahia et al., 2022). Alternative methods, such as high hydrostatic pressure (Georget et al., 2015), or ultrasound or pulsed electric field treatments (Uemura et al., 2009), have been reported to be effective, but are often hindered by associated costs (Putnik et al., 2018). To overcome the above limitations and to be aligned 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 as essential oil components (EOCs). These compounds possess substantial broad-spectrum antimicrobial activity. Their use as preservatives is increasing, especially because many of these components are recognized as Generally Recognized 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 present drawbacks, such as flavor modification, strong smell or low solubility in aqueous media, which justify the need for innovative solutions. To address these issues, the covalent immobilization 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; 2019b; Ribes et al., 2017 and 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 immobilized on silica particles to control the growth of different *Alicyclobacillus acidoterrestris* strains and guaiacol production capacity in orange juice by two different approaches: direct contact with juice in uniform dispersion and filtering juice through a layer of functionalized particles.

2. Materials 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). Orange juice was bought from a local supermarket. The five *A. acidoterrestris* strains used in this study were sourced from a private collection, previously isolated from commercial samples of orange juice (st1, st2, st3 and st4) and pear juice (st5). These strains were isolated and characterized 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 utilized 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 respectively characterized as the minimum concentration at which bacterial growth was no longer evident and at which no microorganism growth was observed by plate count (Owuama et al., 2017).

Before the study began, strains were thawed and reconstituted from cryovials according to the procedure reported by Tedeschi and De Paoli (2011). Bacteria 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 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 characterization of the EOC-functionalized 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 functionalized with the compounds in a two-step procedure. First, 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 (11000 *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. 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. Afterward, reactions were stirred for 24 h at 60 °C, the solvent was removed by centrifugation (11000 *g*) and particles were washed with distilled water until the leaching of EOCs and formaldehyde were 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 functionalized particles was routinely analyzed 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 x 4.6 mm). The UV detection wavelengths for Eu, formaldehyde and Thy were set at 280 nm, 360 nm and 277 nm, respectively.

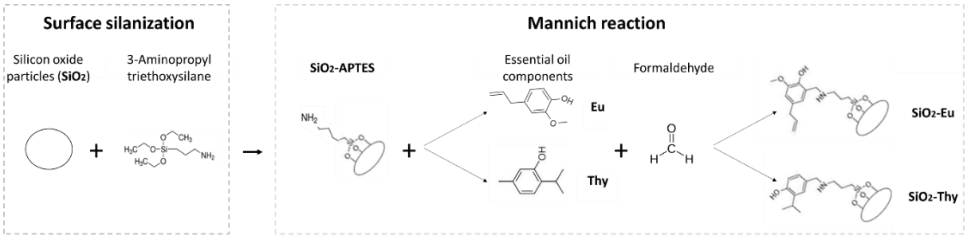

Figure 2. Mannich reaction scheme and reagents for the immobilization of eugenol (Eu) and thymol (Thy) on silicon oxide particles of size of 5-25 µm and 50-110 µm.

The characterization of the **SiO_2** and EOCs-functionalized silica particles (**SiO_2-EOCs**) involved standard techniques, which were run to assess both morphology and the degree of functionalization. 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. The degree of functionalization (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 immobilized EOCs

The effect of the applications of the EOCs-functionalized 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 immobilized EOCs (**SiO_2-EOCs**), the equivalent to the free EOCs, were calculated from the degree of functionalization 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 Determination of viable cell counts

The viable *A. acidoterrestris* cell counts after both treatments were quantified by the plate count technique on BAT agar. To do so, each sample was 10-fold diluted in peptone water and bacterial colonies were enumerated after 96 h of incubation at 46 °C. 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 of incubation at 46 °C. 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 x 4.6 mm) was employed. The mobile phase, which consisted of 66.5 % deionized water, 3.5 % formic acid and 30% methanol for 15 minutes, 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 growth of bacteria ($\log_{10}$) and guaiacol production (mg/L) was statistically analyzed 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 bacteria 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 and 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.

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^{bB}	> 10	$> 10^{D}$	> 10	2.5^{cA}	> 10	5^{bC}	> 10
St2	5^{bB}	> 10	$> 10^{C}$	> 10	1^{bA}	> 10	5^{bB}	> 10
St3	3.75^{bB}	> 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	$> 10^{C}$	> 10	0.75^{aA}	> 10	3.75^{aB}	> 10

**Different small letters in the same column indicate significant differences among strains. Different capital letters in the same row denote significant differences among treatments (p<0.001).*

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 vegetative *A. acidoterrestris* cells. 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 bacteria growth. For Ger, no antimicrobial activity was observed against any of the evaluated strains, which would explain the why relevant studies into this EOC are lacking. 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 of 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 (Silva et al., 2015 and Pornpukdeewattana et al., 2020).

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.

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, **Figure 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 rate, approximately 5 log_{10} cycles, when antimicrobial treatment was lacking ($p > 0.05$).

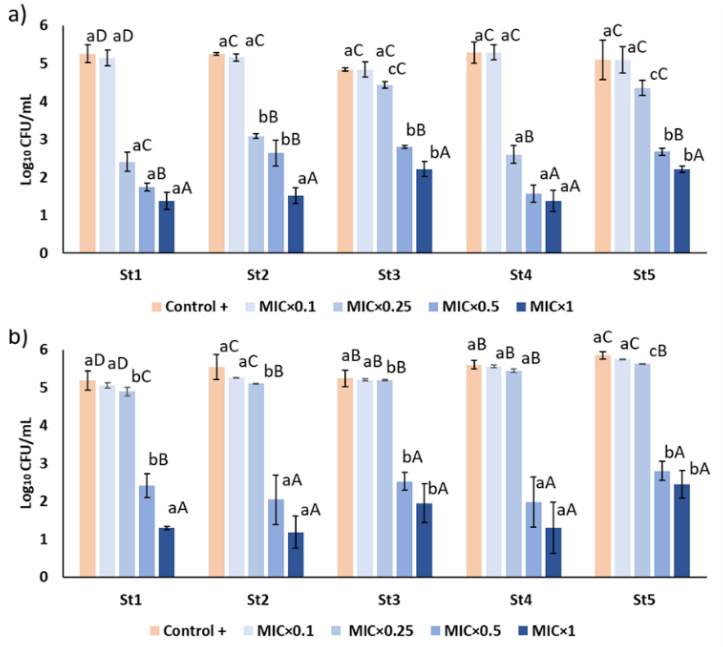

Figure 3. Effect of the different treatments of free a) Eu and b) Thy at MIC×0.1, MIC×0.25, MIC×0.5 and MIC×1 on the growth of the different A. *acidoterrestris* strains (St1, St2, St3, St4, St5). Mean value±SD (n=3). *Different small letters indicate significant differences in the A. acidoterrestris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

Regarding the free Eu application, **Figure 3A** shows that the application of MIC×0.1 did not impact bacteria growth because similar counts to the control were found ($p>0.05$). At MIC×0.25 of Eu, bacteria 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 (**Figure 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, that would favor the effect of EOCs (Gómez-Llorente et al., 2024a).

In parallel to the study into the effect of natural antimicrobials on reducing bacteria 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 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).

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		77.01±3.21^{bC}	76.47±2.61^{bC}	n.d^{aA}	n.d^{aA}	n.d^{aA}
St2		61.92±1.32^{aB}	58.06±3.29^{aB}	n.d^{aA}	n.d^{aA}	n.d^{aA}
St3	Eu	94.68±4.71^{cB}	92.41±3.89^{cB}	23.67±3.65^{bB}	n.d^{aA}	n.d^{aA}
St4		84.48±4.56^{bB}	85.98±6.74^{bB}	n.d^{aA}	n.d^{aA}	n.d^{aA}
St5		97.16±0.98^{cC}	95.29±4.98^{cC}	51.17±2.35^{cB}	n.d^{aA}	n.d^{aA}
St1		74.61±1.14^{bC}	71.18±2.01^{bC}	70.27±2.76^{cB}	n.d^{aA}	n.d^{aA}
St2		63.45±3.19^{aB}	65.49±4.31^{aB}	51.87±2.98^{aB}	n.d^{aA}	n.d^{aA}
St3	Thy	90.59±6.79^{cB}	92.36±2.47^{cB}	60.02±1.98^{bB}	n.d^{aA}	n.d^{aA}
St4		86.84±3.41^{cB}	87.65±5.19^{cB}	60.61±2.49^{bB}	n.d^{aA}	n.d^{aA}
St5		95.05±4.40^{cB}	91.48±3.47^{cB}	90.47±2.42^{dB}	n.d^{aA}	n.d^{aA}

** Different small 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 ($p<0.001$).*

Finally, the relation between antimicrobial activity (**Figure 3**) and guaiacol inhibition (**Table 2**) for the treatments with free Eu and Thy was analyzed. At MIC×0.5, both antimicrobials effectively inhibited guaiacol formation, but the viable bacteria counts which 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 bacteria 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 bring about changes in enzymes, such as dehydrogenase or decarboxylase involved in guaiacol metabolic pathways (Cai et al., 2015 and Wahia et al., 2022).

3.3 *In vivo* effect of incorporating immobilized 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 bacteria growth and to inhibit guaiacol production, the next step was to assess if this reduction could be achieved using the EOCs immobilized 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 functionalized with Eu and Thy.

3.3.1 Characterization of the functionalized silica particles

The functionalized silica particles were characterized using FESEM and an elemental analysis to corroborate the efficiency of the functionalization procedure based on the Mannich reaction. **Figure 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 immobilization 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. (2024a), 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 functionalization.

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 functionalization results indicated that the **SiO_2-EOCs** used in this work were properly functionalized.

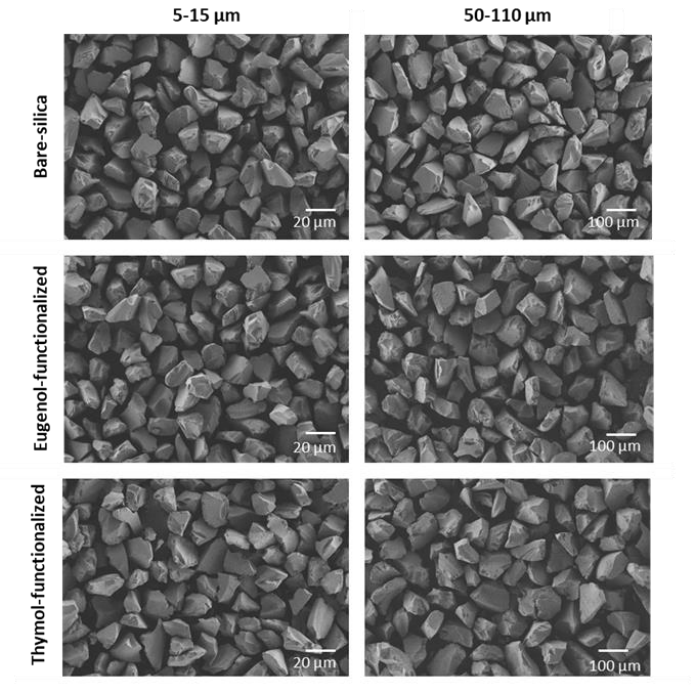

Figure 4. FESEM images of 5 to 15 µm (left column) 50-110 µm (right column) for both the bare (**SiO_2**) and silica particles functionalized with eugenol (**SiO_2-Eu**) and thymol (**SiO_2-Thy**).

3.3.2 Treatment based on adding functionalized particles to juice

The fist approach to employ the functionalized 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. **Figure 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. (2019a) against *Escherichia coli*. In contrast, the immobilization of the different EOCs onto silica particles reduced bacteria 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 treatments with **SiO_2-Thy**, a significant reduction of approximately 3 log_{10} was observed after applying MIC×1 of functionalized particles. These findings indicate less antimicrobial activity for **SiO_2-Thy** compared to **SiO_2-Eu**.

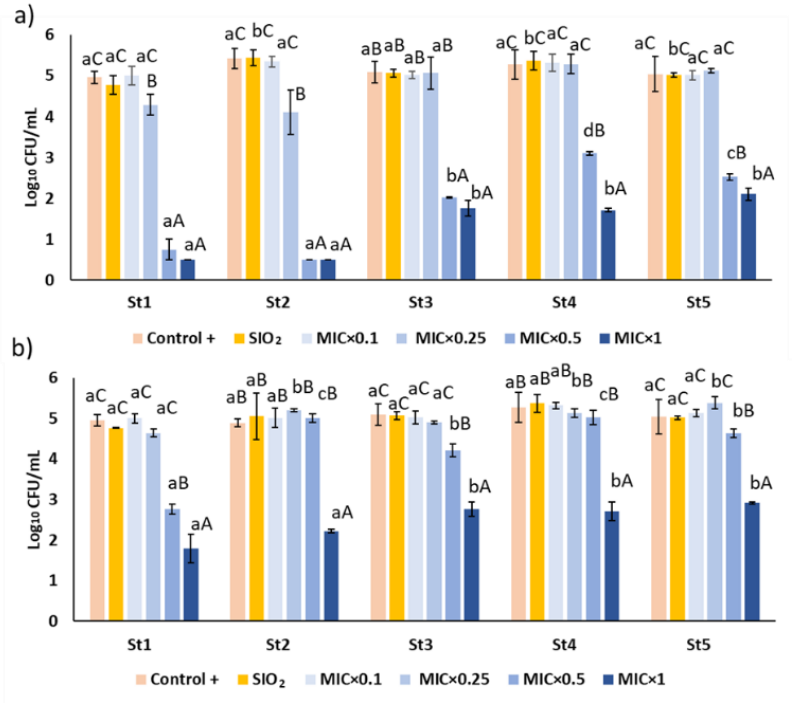

Figure 5. Effect of the various treatments of a) **SiO_2-Eu** and b) **SiO_2-Thy** at MIC×0.1, MIC×0.25, MIC×0.5, MIC×1 against the different *A. acidoterrestris* strainf (St1, St2, St3, St4, St5). Mean value±SD (n=3). *Different small letters indicate significant differences in the A. acidoterrestris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

Despite studies not having specifically focused on evaluating the antimicrobial properties of immobilized 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 functionalized with Eu against *Escherichia 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 immobilized 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-functionalized 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 flavor (Gómez-Llorente et al., 2023).

When comparing the effects of the free and immobilized EOCs on the growth of different strains (see **Figure 3** and **Figure 5** respectively), distinct patterns emerged. First, both the free and immobilized forms of EOCs resulted in significant reductions when applying MIC×0.5 of the antimicrobial. Second, of all the treatments, applying MIC×1 of **SiO_2-Eu** achieved the greatest reduction in microorganism growth. This suggests that the immobilization of EOCs enhances their activity, possibly due to the higher local concentration of the antimicrobial achieved after being immobilized 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, as antimicrobial activity was lacking, **SiO_2** did not inhibit guaiacol production in any of the evaluated strains ($p>0.05$). On the contrary, the treatment with the functionalized 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 complete guaiacol production inhibition for both the tested antimicrobials. Despite complete guaiacol inhibition, the same antimicrobial concentration did not completely reduce microorganism growth (see the details in **Figure 5**), which suggests that sublethal concentrations of EOCs would inhibit guaiacol production by the different *A. acidoterrestris* strains. When analyzing the 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.

Table 3. Effect of the different **SiO_2-Eu** and **SiO_2-Thy** treatments at MIC×0.1, MIC×0.25, MIC×0.5 and MIC×1 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		77.43±6.37^{bD}	76.93±3.62^{bD}	70.74±1.28^{bC}	48.79±2.72^{bB}	n.d^{aA}	n.d^{aA}
St2		60.14±7.19^{aC}	59.31±2.64^{aC}	55.22±0.63^{aC}	35.17±7.87^{aB}	n.d^{aA}	n.d^{aA}
St3	Eu	91.87±3.04^{cD}	90.99±2.93^{cD}	77.66±5.84^{aC}	57.66±5.84^{bB}	n.d^{aA}	n.d^{aA}
St4		81.05±6.43^{bC}	79.34±5.79^{bC}	68.27±5.41^{bB}	62.44±5.08^{dB}	n.d^{aA}	n.d^{aA}
St5		92.66±5.11^{cD}	92.12±1.65^{cD}	72.93±5.50^{bC}	54.38±0.97^{bB}	n.d^{aA}	n.d^{aA}
St1		76.32±2.65^{bC}	72.51±4.53^{bC}	71.66±1.75^{bB}	69.53±2.19^{cB}	n.d^{aA}	n.d^{aA}
St2		67.47±3.76^{aC}	67.47±3.76^{aC}	60.79±2.87^{aC}	53.49±5.31^{aB}	n.d^{aA}	n.d^{aA}
St3	Thy	91.80±1.99^{dD}	89.12±1.92^{dD}	83.58±4.94^{cC}	63.53±2.19^{bB}	n.d^{aA}	n.d^{aA}
St4		84.30±2.09^{cB}	84.10±4.21^{cB}	72.74±3.75^{bB}	58.87±4.75^{aB}	n.d^{aA}	n.d^{aA}
St5		94.53±6.99^{dD}	90.31±7.02^{dD}	83.12±4.63^{cC}	67.98±0.82^{cB}	n.d^{aA}	n.d^{aA}

** Different small 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 ($p<0.001$).*

To compare the effect of the strain, the type of EOC, the concentration and the dosage 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 dosage form (free or immobilized) had a significant effect on both microbial growth and guaiacol inhibition. These results are very interesting because the immobilization process maintained the antimicrobial activity of the evaluated EOCs, while offering significant advantages, such as a lower impact on the sensory profile (Ribes et al., 2017), easy dosing, as well as the possibility of removing active ingredients by centrifugation or filtration, and then reusing them in a subsequent batch.

Table S1. F-ratio values and p-values obtained in the multifactor ANOVA for microbial growth (log_{10}) and the guaiacol concentration (mg/L) after treatment with the different essential oil components.

	Microbial growth		Guaiacol	
	F-ratio	***p-value***	***F-ratio***	***p-value***
A: Strain	4.55	0.0030	15.48	0.0000
B: EOC	25.88	0.0000	20.31	0.0000
C: Concentration	128.65	0.0000	539.21	0.0000
D: Dosage form	3.86	0.0545	0.53	0.4712
Interactions				
AB	0.26	0.9026	0.31	0.8718
AC	0.72	0.7660	3.06	0.0010
AD	1.42	0.2406	1.28	0.2900
BC	3.97	0.0067	15.80	0.0000
BD	1.35	0.2500	4.34	0.0419
CD	2.34	0.0658	7.63	0.0001

3.4 Treatment based on juice filtration though functionalized particles

Having demonstrated that the immobilized EOCs effectively reduced growth and inhibited guaiacol production in *A. acidoterrestris* by coming into contact with agitation that involved a 24-hour incubation period, we further investigated the potential of **SiO_2-Eu** and **SiO_2-Thy** as filtering aids (García-Ríos et al., 2018 and 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 **Figure 5,** respectively.

Table 4 shows the bacteria 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 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}.

Table 4. Effect of the different **SiO_2-Eu** and **SiO_2-Thy** treatments 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	Guaiacol
Control	5.01 ± 0.29^{C}	95.83 ± 6.02^{B}
SiO_2	4.83 ± 0.18^{C}	94.37 ± 4.99^{B}
Eu	0.50 ± 0.00^{A}	$n.d^{A}$
Thy	3.43 ± 0.46^{B}	$n.d^{A}$

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

The antimicrobial activity of the functionalized silica particles with 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-functionalized silica particles, along with complete indigenous juice microbiota removal, including mesophilic, psychrophilic bacteria, and mold/yeast. This resulted in the microbial stabilization of juice on the first 120 refrigerated storage days. Peña-Gómez et al. (2020) also examined the effect of immobilized 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 immobilized 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 (Hyldgaard et al., 2012; Gómez-Llorente et al.,2024a).

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 immobilized 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 immobilization of EOCs onto silica surfaces would offer a viable solution to address solubility issues and the undesirable odors or flavors that result from EOCs application (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 should also be indicated that consumer perceptions are more favorable toward 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 immobilized on silica particles to reduce *Alicyclobacillus acidoterrestris* growth and to inhibit guaiacol production in orange juice. The results reveal that the immobilization 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 immobilization 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 the application of free EOCs, such as alterations to food products' odor and taste. Finally, shorter particle contact by the filtration process through the Eu- and Thy-functionalized 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 their prospective industrial applicability 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 physic-chemical alterations and it could, thereby, address the current serious food waste problem.

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6. CHAPTER III

Effect of immobilized EOCs against viruses

6.1. Tulane virus disinfection of drinking water by using natural antimicrobials immobilised on silica particles

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Abstract

A batch process, based on a physico-chemical interaction between Tulane virus (TuV) in water and essential oil components immobilised onto silica microparticles (**SiO_2-EOCs**), was investigated. For this purpose, the **SiO_2-EOCs** were uniformly dispersed in the TuV water sample for 2 h. To assess the treatment effectivity, the infectious virus particles and TuV genomic copies (GCs) with and without treatment were quantified by $TCID_{50}$ and RT-qPCR, respectively. The results indicate that immobilisation of natural antimicrobials markedly increased their antiviral activity. For vanillin and eugenol, the treatment with 20 mg/mL of the functionalised particles reduced TuV infectivity with >5 $\log_{10}$, and with ca. 4 $\log_{10}$ for carvacrol and thymol. The free form of EOCs, however, reduced $TCID_{50}$ with ca. 1 $\log_{10}$. The RT-qPCR analysis showed an effect beyond TuV entrapment on the functionalised silica particles. RNase treatment of samples prior to RNA extraction and RT-qPCR verified that immobilised EOCs lowered TuV infectivity by reducing the integrity of the virus capsid. These results demonstrate the capacity of the immobilised EOCs to accomplish the stringent disinfection requirements for public water systems.

Keywords: eugenol, vanillin, water disinfection, infectivity, capsid integrity**.**

1. Introduction

Safe water is crucial to support main daily activities, such as drinking, irrigating crops or industrial production. Of all the physico-chemical and biological agents that can cause waterborne diseases, viruses are considered the most critical because of the low doses needed to induce disease (Gibson, 2014). In line with this, Le Pendu and Ruvoën-Clouet (2020) stated that waterborne viruses like human noroviruses (HuNoVs) are the key agents that cause the commonest disease: gastroenteritis (Gibson, 2014).

HuNoVs constitute a genetically highly diverse group of viruses in the Caliciviridae family, characterised by being non-enveloped, with an icosahedral capsid and a positive single-stranded RNA genome (Karst and Goodfellow, 2015). Traditionally, HuNoVs are inactivated in water by the application of ozone (Wang et al., 2015) or chlorine (de Abreu Corrêa et al., 2012). However, these processes may generate hazardous disinfection by-products (Richardson et al., 2007). This drawback, and the need to provide safe water, have led to alternative technologies like UV radiation to cause microbial photodegradation (Piccini et al., 2009), ultrafiltration to remove microorganisms larger than the porous size (Matsushita et al., 2013) or nanomaterials able to adsorb viruses onto their surface (Castro-Mayorga et al., 2017). However, the aforementioned techniques have some drawbacks, such as maintenance requirements (Peña-Gómez et al., 2019), lack of specific effect against certain microorganisms (Zhu et al., 2005) or the possible toxicological risks associated with artificial nanostructured materials (Guo et al., 2021).

Another emerging approach to fight waterborne viruses consists of using natural essential oil components (EOCs), such as carvacrol, eugenol, thymol and vanillin (Sánchez and Aznar, 2015 and Sánchez et al., 2015). One of their main advantages is that they are generally recognised as safe (GRAS) (Manion and Widder, 2017). However, despite their reported good antimicrobial effects, employing EOCs in water has significant limitations, such as poor solubility (Ruiz-Rico et al., 2017), contribution of a strong flavour and smell (Walton et al., 2003) and high volatility (Nerio et al., 2010). To overcome these drawbacks, which could limit their use in

the food industry or in water purification processes, EOCs immobilisation onto nano- and microparticles has been proposed (Ruiz-Rico et al., 2017). This approach consists of covalently anchoring EOCs on to the surface of silica or cellulose structures (Ruiz-Rico and Barat, 2021). Their efficient capacity to remove or inactivate foodborne bacteria from liquid or solid food matrices has been recently reviewed (Gómez-Llorente et al., 2023). This immobilisation also increases the antimicrobial activity of EOCs, while preventing their leaching to the matrix (Peña-Gómez et al., 2019) and reducing their sensory impact (Ribes et al., 2017). Although this is a promising technique, most studies have focused on antibacterial activity and some on antifungal activity (Ribes et al., 2017 and Ribes et al., 2019). As no research into the antiviral activity of EOCs-functionalised supports is available, the present work aimed to look into this topic. In comparison to traditional methods, the proposed methodology would not leave any type of residues since the SiO_2-EOCs are removed after treatment. Moreover, it includes natural antimicrobials and avoids the formation of hazardous disinfection by-products. Finally, as a possible substitute to thermal treatment, it would also reduce the carbon footprint of the process (Gómez-Llorente et al., 2023).

With this backdrop, the effect of this disinfection method on HuNoVs is extremely interesting. However, as there are not simple and robust cell culture system for HuNoVs (Farkas et al., 2019), systematic studies that employ these viruses are complex. To solve this problem, the use of Tulane Virus (TuV) as a HuNoVs surrogate has been proposed ever since Li et al. (2013) stated that TuV is genetically close and recognises the same HuNoVs receptors. This virus was discovered in 2008 at the Tulane National Primate Research Center. TuV infects monkey kidney cells (LLC-MK2) with a rapid replication cycle and a visible cytopathic effect (CPE) noted 24 h after inoculation (Farkas et al., 2008).

The goal of this study was to assess the capacity of free and immobilised EOCs to reduce TuV infectivity, and to identify the mechanism of any antiviral effect.

2. Materials and methods

2.1. Chemicals

Carvacrol (≥ 98 % w/w), eugenol (99 % w/w), thymol (≥ 98.5 % w/w), vanillin (> 99 % w/w), (3-Aminopropyl) triethoxysilane (APTES), paraformaldehyde, triethylamine, 2-butanone, chloroform, sodium borohydride, potassium hydroxide (KOH) and silica particles (5–15 µm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, dichloromethane, ethyl alcohol, 2-propanol, sodium chloride, potassium chloride, disodium phosphate heptahydrate, potassium phosphate monobasic, magnesium sulphate ($MgSO_4$), sulphuric acid (H_2SO_4) and hydrochloric acid (HCl) were obtained from Scharlab (Barcelona, Spain). Foetal bovine serum (FBS), Medium 199/Earle's salts/GlutaMAX, and RNase A (10 mg/mL) were purchased from Thermo Scientific/Gibco (Waltham, MA, USA). Penicillin Streptomycin and l-glutamine were acquired from Life Technologies (Paisley, Scotland).

2.2. Functionalisation and characterisation of the silica EOCs particles

Commercial silica particles (**SiO_2**), as an inert support, were functionalised with EOCs according to the method proposed by Ruiz-Rico et al. (2017). Firstly, carvacrol (Car), eugenol (Eu), and thymol (Thy) were converted into aldehyde derivatives to keep their hydroxyl group (-OH) free, which is crucial for maintaining antimicrobial activity. In this way, the derivatised aldehyde groups react with the amine group of APTES instead of with the hydroxyl group.

Eugenol aldehyde was synthesised following a Reimer-Tiemann route. In particular, 150 mL of water was heated to 80 °C in a round-bottomed flask. Then 22 mmol of Eu was dissolved, the temperature decreased to 60 °C, and 400 mmol of KOH and 88 mmol of chloroform were added. The solution was mixed for 24 h and acidified with 50 mL of 10 % H_2SO_4. The aldehyde derivative (organic phase) was extracted using 2-butanone and concentrated with low pressure at room temperature (RT). The carvacrol (Car) and thymol (Thy) aldehydes were synthesised by direct formylation. In a typical synthesis, 40 mmol of both Car or Thy were mixed with 150 mL of acetonitrile, 150 mmol of triethylamine and 40 mmol of $MgSO_4$

inside a round-bottomed flask in an argon atmosphere for 15 min at RT. Afterwards, 270 mmol of paraformaldehyde was added, and the reaction was refluxed at 83 °C for 3.5 h. After lowering the temperature to RT, 300 mL of 5 % HCl was added. The aldehyde derivatives were extracted with diethyl ether and finally concentrated at low pressure. For vanillin (Va), the presence of an aldehyde group rendered this last step unnecessary.

Secondly, these aldehydes were treated with APTES to create the corresponding alkoxysilane derivatives. For that purpose, 2 mL of the EOCs aldehyde derivatives were reacted with 2.3 mL of APTES under reflux for 1 h at 60 °C in 20 mL of dichloromethane. Then, the solvent was evaporated at low pressure at RT. To anchor the four alkoxysilane derivatives to the silica particles, 1 g of the support was suspended in 20 mL of 2-propanol. Thereafter, the alkoxysilane derivatives were added and stirred for 3 h at RT. The functionalised particles were washed by centrifugation using water and 2-propanol, and were finally vacuum dried for 24 h to obtain the **SiO_2-Eu**, **SiO_2-Car**, **SiO_2-Thy** and **SiO_2-Va** solids.

The **SiO_2** and EOCs-functionalised silica particles (**SiO_2-EOCs**) were characterised by standard techniques to determine the morphology and degree of functionalisation. The morphological analysis was performed by field emission scanning electron microscopy (FESEM) using a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany), observed in the secondary electron mode. Finally, the degree of functionalisation (mg EOC/g SiO_2) was calculated from the elemental analyses for C, H and N with a Vario EL III Element Analyser (Elemental Analyses System GMHB, Langenselbold, Germany) (Ruiz-Rico et al., 2017).

2.3. Propagation of Tulane virus

Tulane virus (TuV) strain M033 was provided by T. Farkas (Louisiana State University at Baton Rouge, LA, USA). The virus was cultured in LLC-MK2 cells (ATCC CCL-7) at 37 °C and 5 % CO_2 in Medium 199/Earle's salts/GlutaMAX with 10 % FBS and 1 % Penicillin Streptomycin.

Confluent cells were inoculated with TuV at a multiplicity of infection of 1 in medium without supplements (maintenance medium). After 3 days, TuV was harvested by 3× freeze-thawing and debris was removed by centrifugation at 2,500 ×g for 5 min. Finally, TuV was aliquoted and stored at −80 °C until use.

2.4. Quantification of the infectious TuV

Virus titration was performed in 96-well plates seeded with 10^4 LLC-MK2 cells per well following the Spearman-Karber method (Ramakrishnan, 2016). Serial 10-fold dilutions (50 µL) of the virus samples were added to four parallel wells with a confluent cell layer. Cells were incubated at 37 °C for 2 h in 5 % CO_2. Then 150 µL maintenance medium was added and the CPE was read after 5 days using an inverted optical microscope. The virus titre is given as tissue culture infectious dose 50 ($TCID_{50}$).

2.5. Quantification of the TuV genome copies

For RNA extraction, 500 µL of sample was added to 2 mL of NucliSENS miniMAG Lysis Buffer (Biomerieux, Marcy l'Etoile, France) and RNA was extracted according to the manufacturer. RNA was eluted in 100 µL elution buffer and stored at −80 °C before use.

The TuV genome copies (GCs) were quantified in our stock sample using RT-ddPCR and employing the same protocol as reported by Stoppel et al. (2023). The TuV stock contained 4.6×10^8 GC/mL.

For the antiviral study, RT-qPCR was used for the relative quantification of GCs. The TuV primers and probe were: TVIF_f (5'-CTGGGATACCCACAACATC-3'), TVIF_r (5'-GCCAGTTAACAGCTTCAGC-3') and TVIF_probe (5'-FAM-TGTGTGTGCCACTGGATAGCTAGCACCBHQ-3') (Drouaz et al., 2015). RT-qPCR was performed with the TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems™, CA, USA) in accordance with the manufacturer's protocol. The total reaction volume was 20 µL, which contained 3 µL of RNA and 10 µM of each primer and probe. Cycling conditions were 50 °C for 5 min, 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s and 64 °C for 40 s. Reactions were run using the Stratagene

AriaMx Real-Time PCR System (Agilent Technologies, Inc., USA). Data are presented in cycle threshold (Ct) values, defined as the number of amplification cycles needed for fluorescence to cross a certain baseline (Grosdidier et al., 2017).

Using the Ct values and the efficiency (E) of RT-qPCR, the relative quantification of genomes was performed by applying the following formula (Christensen et al., 2017):

$$Ns = Nc \cdot (1 + E)^{(Ct_c - Ct_s)}$$

where Ns and Nc are the TuV concentration (GC/mL) in the sample and control (TuV stock), respectively.

E (0.92) was estimated from a standard curve made from the 10-fold and 4-fold serial dilutions of the TuV RNA.

2.6. Testing the cytotoxicity of the free and immobilised EOCs

In order to assess the possible cytotoxicity of free EOCs, each EOC (60 mM) was stirred at 37 °C for 2 h in phosphate-buffered saline (PBS) containing 1 % ethyl alcohol to be completely dissolved. All the samples were filtered through cellulose filter (0.45 µm pore size). Several dilutions (1,5, 1,10, 1:15, 1:20, 1:60, 1:75 and 1:100) were prepared in the cell culture maintenance medium. Dilutions (50 µL) were inoculated onto LLC-MK2 cells, which were then incubated for 5 days and analysed for the CPE. PBS and the PBS with 1 % ethyl alcohol were employed as negative controls. The treatments were done 3 times in triplicate (n = 9).

In parallel, the cytotoxicity of any leaching of **SiO_2** and **SiO_2-EOCs** was tested on LLC-MK2 cells. For this purpose, for each of the EOCs, the non-cytotoxic concentrations determined in the free form was taken as a reference. Then, the equivalent concentration of the immobilised EOCs (**SiO_2-Car**, **SiO_2-Eu**, **SiO_2-Thy** and **SiO_2-Va**) was calculated from the degree of functionalization (**Table 1**) (Ruiz-Rico et al., 2017).

Table 1. Degree of functionalisation according to essential oil components (EOCs) content.

Support	mg EOC/g SiO_2
SiO_2-Eu	25.3 ± 0.2
SiO_2-Car	8.2 ± 0.1
SiO_2-Thy	10.0 ± 0.1
SiO_2-Va	68.9 ± 0.3

2.7. Water treatment for TuV removal

Experiments for the TuV removal through free EOCs, **SiO_2-EOCs**, and **SiO_2** were carried in using batch approach based on Guo et al. (2023) with some modifications. To assure the homogeneity of the water sample, distilled water in PBS inoculated with TuV ($6.14 \cdot 10^6$ $TCID_{50}$/mL) was used to simulate a water sample in which suspended solids and organic matter would have been removed (Sahakijpijarn et al., 2019 and Zani et al., 2015).

Samples were then treated with the free EOCs, **SiO_2** and **SiO_2-EOCs** at 37 °C for 2 h with shaking (1,000 g) to maintain a uniform dispersion of particles within the solution.

After incubation, all the samples were filtered using the 0.45 µm nylon filter to separate the silica particles. The concentration of the free EOCs was selected according to the cytotoxicity assay. The equivalent concentrations of **SiO_2-EOCs** were established according to their degree of functionalisation (Ruiz-Rico et al., 2017).

The SiO_2 concentration was 65 mg/mL as it was the maximum concentration tested. The positive control (non-treated) consisted of TuV in PBS with 1 % of ethyl alcohol, as used when testing the antiviral efficacy of free EOCs.

The effect of **SiO_2**, the free EOCs and **SiO_2-EOCs** on TuV was assessed by three different approaches: by $TCID_{50}$ to quantify infectious virus particles, and by RT-qPCR to quantify GCs, w/wo, prior to RNase treatment. RNase treatment was

performed to assess the degree of TuV capsid degradation from the different treatments.

The infectious TuV was quantified by the $TCID_{50}$ procedure described in Section 2.4.

The TuV GCs were quantified by RT-qPCR (Section 2.5) before and after treating samples with 10 µL of RNase A (10 mg/mL) at 37 °C for 30 min. All the treatments were done 3 times in triplicate (n = 9).

2.8. Statistical analysis

Statistical data processing was carried out by Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). Data were tested by a one-way ANOVA to check for significant differences among the EOC types and concentration with a 95 % confidence interval ($p < 0.05$).

3. Results and discussion

3.1. Characterisation of materials

Four EOC-functionalised supports were prepared and characterised before evaluating their antiviral activity against TuV. Fig. 1 shows the morphology of **SiO_2** and **SiO_2-Eu** using FESEM. The silica particle size was 5–15 µm. No difference between the support surface was detected when comparing **SiO_2** and **SiO_2-Eu**, which confirmed that the immobilisation process did not affect support integrity. Equivalent results were found after the immobilisation of the other EOCs (images not shown).

The amount of EOCs attached to the surface of the silica particles was determined by an elemental analysis. As shown in Table 1, the degree of functionalisation ranged from ca. 10 mg/g SiO_2 for Car or Thy, to 70 mg/g SiO_2 for Va. These results suggest that Va, which originally has an aldehyde group, was anchored to the SiO_2 particles to a greater extent than the other phenols, which must be derivatised to exhibit an aldehyde group, used as a linker to the APTES molecule.

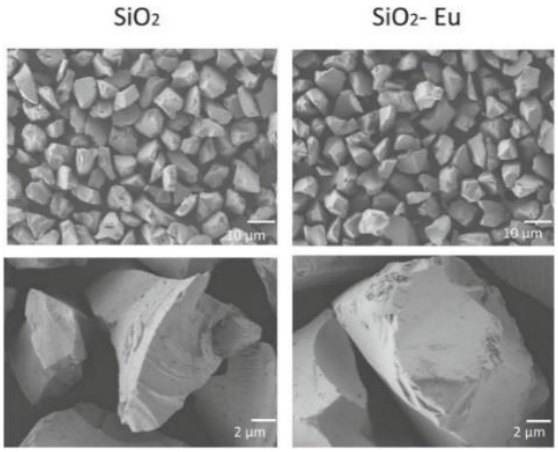

Figure 1. FESEM images of the bare (left) and eugenol- (right) functionalised silica particles.

This agrees with previous studies, which have used the same functionalisation routes of EOCs anchored onto amorphous silica, where **SiO_2-Va** and **SiO_2-Eu** reactions obtained the biggest functionalisation yields (Ribes et al., 2021 and Ruiz-Rico et al., 2017).

3.2. Cytotoxicity of the free EOCs

As an initial study on cytotoxicity, the LLC-MK2 cells were exposed to different concentrations of the free EOCs. Cells revealed damage in a concentration-dependent way. The maximum non-cytotoxic concentration of Eu, Car and Thy was established as 1 mM, with 3 mM for Va. Regarding the solvent control, the PBS with 1 % ethyl alcohol had no cytotoxic effect. In line with these findings, Fuentes et al. (2021) found that 1 mM Eu and 3 mM Va had no cytotoxic effect on HpeG2 cells.

After confirming the maximum non-cytotoxic concentration of EOCs in the free form, **SiO_2** and **SiO_2-EOCs** were evaluated at equivalent concentrations. For this calculation two assumptions should be considered. On the one hand, the degree of functionalization calculated through elemental analysis. On the other hand, the fact that the sample could be treated with 10-fold of the highest non-cytotoxic concentration, since the treated water sample is diluted 1:10 before being added to the cells (see Section 2.4 for details).

Accordingly, 10 mM of Eu, Car and Thy was equivalent to ca. 65, 180 and 150 mg/mL of **SiO_2-Eu**, **SiO_2-Car** and **SiO_2-Thy** respectively, while 30 mM of Va was equivalent to ca. 65 mg/mL of **SiO_2-Va**. On the other hand, the highest concentration properly suspended in media was 65 mg/mL (data not shown). Therefore, 65 mg/mL of **SiO_2** and **SiO_2-EOCs** was the maximum scope of the study.

No cytotoxic effects in LLC-MK2 were found at 65 mg/mL for **SiO_2** or each of the tested **SiO_2-EOCs**. Similar results were obtained by Fuentes et al. (2021) when exposing cells to the media that had been in contact with carvacrol and thymol functionalised silica particles.

3.3. Water treatments for TuV removal

3.3.1 Water treatment with free EOCs

The treatment of TuV with Car, Eu and Thy 10 mM showed no antiviral effect (**Table 2**). In contrast, the Va treatment at 30 mM gave a TuV $TCID_{50}$ reduction of 1.38 $\log_{10}$ ($p < 0.05$). The greater Va antiviral activity was probably caused by the higher applied dose due to lower cytotoxicity (Section 3.2). Previous works have evaluated the effect of EOCs on different HuNoV surrogates. In a study with feline calicivirus, Sánchez and Aznar (2015) tested the effect of Thy and found that 33.3 mM was needed to reduce infectivity from 5.88 to <2.13 $\log_{10}$ $TCID_{50}$/mL. The same Car concentration was also needed to decrease the infectivity of feline calicivirus and murine norovirus from 5.82 and 5.70 to <1.32 and < 2.13 $\log_{10}$, respectively (Sánchez et al. 2015). Gilling et al. (2014) evaluated the antiviral activity of non-immobilised Car against murine norovirus and found that 33.3 mM reduced the virus infectivity from 6 to <1.48 $\log_{10}$ $TCID_{50}$/mL. The higher antiviral activity reported by these authors was due to the higher concentrations tested in those studies.

Table 2. Infectious TuV particles (log_{10} $TCID_{50}$/mL) after treatment with the free essential oil components (EOCs). Mean values ± SD (n = 9).

	Mean log_{10} $TCID_{50}$/mL	
Treatment	**Virus titre Mean ± SD**	**Virus Reduction**
Control	6.42 ± 0.38[a]	–
Car (10 mM)	6.05 ± 0.43[a]	0.37
Eu (10 mM)	6.29 ± 0.51[a]	0.13
Thy (10 mM)	6.17 ± 0.43[a]	0.25
Va (30 mM)	5.04 ± 0.52[b]	1.38
α	*	

Different letters indicate statistically significant differences in TuV $TCID_{50}$/mL among the applied EOCs. Significance level p < 0.05.

3.3.2. Water treatment with SiO2 and SiO2-EOCs

After studying the water treatment with free EOCs, the effect of SiO_2 and **SiO_2-EOCs** was evaluated. **Table 3** shows the number of the TuV infectious particles after incubation with SiO_2 and SiO_2-EOCs. As it shows, the treatment with **SiO_2-Eu** and **SiO_2-Va** at 65 mg/mL reduced the TuV infectious particles ($TCID_{50}$) by >5 log_{10}. The reduction achieved by **SiO_2-Car** and **SiO_2-Thy** was 4.5 log_{10}. These findings indicate that, although the free EOCs showed no or very low antiviral activity, an equivalent concentration of the EOCs applied as **SiO_2-EOCs** significantly increased (p = 0.000) the antiviral activity against TuV.

This result is of extremely interesting because the United States Environmental Protection Agency (USEPA, 1991) states that the different processes covered for water treatment after organic matter removal must give a total reduction of at least 4 log_{10}. Therefore, **SiO_2-EOCs** would be effective for water disinfection.

After the initial testing, another study with a lower **SiO_2-EOCs** concentration (20 mg/mL) was carried out. **SiO_2-Eu** and **SiO_2-Va** showed the same reduction in the infectious particles as when using the higher concentration (p > 0.05). However, the treatment with 20 mg/mL of **SiO_2-Car** and **SiO_2-Thy** led to lesser reduction (ca. 2.7 log10) (p = 0.000). In this case, the lower concentration of particles decreased antiviral activity, possibly due to poor contact between **SiO_2-EOCs** and the virus.

Table 3. Infectious TuV particles after incubation with the bare silica particles and silica particles with the immobilised essential oil components at 65 mg/mL and 20 mg/mL. Mean values ± SD (n = 9).

Treatment	Mean log_{10} $TCID_{50}$/mL				Δ reduction (log_{10})	α
	65 mg/mL		20 mg/mL			
	Titre ± SD	Reduction	Titre ± SD	Reduction		
None	6.42 ± 0.20^{a}		6.44 ± 0.05^{a}			ns
SiO_2	6.02 ± 0.12^{a}	0.40	6.16 ± 0.19^{b}	0.28	0.12	ns
SiO_2-Car	1.96 ± 0.65^{bA}	4.46	3.72 ± 0.22^{cB}	2.72	1.74	***
SiO_2-Eu	1.05 ± 0.02^{c}	5.37	1.06 ± 0.07^{d}	5.38	- 0.01	ns
SiO_2-Thy	1.57 ± 0.35^{bA}	4.85	3.66 ± 0.05^{cB}	2.78	2.07	***
SiO_2-Va	1.01 ± 0.01^{c}	5.41	1.07 ± 0.07^{d}	5.37	0.04	ns
α	***		***			

*Δ reduction: titre reduction (infectious virus) between the treatment with 65 mg/mL and that with 20 mg/mL. Different small letters in the same column indicate significant differences in TuV log_{10} $TCID_{50}$/mL between types of particles, while different capital letters in the same row denote statistically significant differences in TuV log_{10} $TCID_{50}$/mL between particle concentrations. Significance levels (α): ns (not statistically significant), *** ($p < 0.001$).*

Comparing the effect of the free and immobilised EOCs on the infectious virus particles showed that anchoring improved the antiviral effect of each EOC. Thus, the immobilisation of EOCs lowered the concentration needed to achieve a significant antiviral effect on TuV. For Eu and Va, the application of one third the immobilised EOCs concentrations, the equivalent to the free forms (3.07 and 9.10 mM, respectively), achieved the complete reduction in infectivity. In addition, **SiO_2-Car** and **SiO_2-Thy** at 20 mg/mL demonstrated that employing one tenth of the equivalent free concentration remarkably lowered the number of the infectious TuV.

The clear difference in the antiviral effect between the free and immobilised EOCs was firstly indicated by Peña-Gómez et al. (2018) when functionalising cellulose with amines against *Escherichia coli*. These authors concluded that the effect could be due to the higher local concentration of active agents when immobilised on supports.

The antiviral effect on the type of EOC was also analysed in the present study. Concretely, 3 mM of Eu (20 mg/mL of **SiO_2-Eu**) showed greater TuV infectivity reduction than 3 mM of Car and Thy (65 mg/mL of **SiO_2-Car** and **SiO_2-Thy**), which suggests that antiviral activity depends mainly on the chemical structure of EOCs. The differences in the antimicrobial activity among EOCs at the same equivalent concentration agreed with Ruiz-Rico et al. (2017) when testing Car, Eu, Thy and Va silica particles against bacteria (*Escherichia coli* and *Listeria innocua*).

Regarding **SiO_2**, a limited effect on the TuV infectious particles was found. Lack of antimicrobial activity of the bare silica particles has also been shown in a previous study (Ruiz-Rico et al.,2017), which confirms that the activity of **SiO_2-EOCs** is due to the anchoring of EOCs onto the surface of SiO_2.

3.4. Antiviral mechanism of the SiO_2-EOCs

After confirming the capability of **SiO_2-Car**, **SiO_2-Eu**, **SiO_2-Thy** and **SiO_2-Va** to reduce the number of the infectious TuV particles, the next step was to assess if this reduction was due to a physical retention of virus particles by **SiO_2-EOCs** and/or by damage to the virus particle. To assess any degree of capsid damage, RNA was extracted w/wo prior RNase treatment.

3.4.1. Quantification of the total virus

In order to quantify the number of the TuV GCs (representing the total of the infectious and non-infectious viruses), samples were analysed by RT-qPCR.

For **SiO_2**, the data showed a non-significant reduction (<1 $\log_{10}$) (**Table 4**). This limited capacity of SiO_2 to bind the virus and other bioactive molecules has also been reported by Sellaoui et al. (2021).

When the virus was treated with **SiO_2-EOCs**, the number of the TuV GCs significantly decreased depending on particle concentration and type of EOC ($p = 0.000$). **SiO_2-Eu** and **SiO_2-Va** gave a marked reduction in the TuV GCs of ca. 4 $\log_{10}$ at both concentrations (20 or 65 mg/mL), while the **SiO_2-Car** and **SiO_2-Thy** treatments with 20 mg/mL displayed lower reductions (ca. 2.10 and 2.4 $\log_{10}$,

respectively). In this case, increasing the particle concentration (ca. 3×) also reduced the number of TuV to a greater extent (p = 0.032 and p = 0.008, respectively).

Table 4. The TuV genome copies (GCs) measured by RT-qPCR after incubation with the bare and essential oil components (EOCs) silica particles at 65 mg/mL and 20 mg/mL. Mean values ± SD (n = 9).

Treatment	Mean log_{10} TuV GC/mL ± SD				Δ reduction (log_{10})	α
	65 mg/mL		20 mg/mL			
	Titre ± SD	Reduction	Titre ± SD	Reduction		
None	7.84 ± 0.04^{a}		7.99 ± 0.15^{a}			
SiO_2	6.98 ± 0.02^{bA}	0.86	7.77 ± 0.15^{aB}	0.22	0.64	***
SiO_2-Car	4.82 ± 0.30^{cA}	3.02	5.69 ± 0.07^{bB}	2.30	0.72	*
SiO_2-Eu	3.30 ± 0.56^{e}	4.54	4.06 ± 0.22^{d}	3.93	0.61	ns
SiO_2-Thy	4.22 ± 0.28^{dA}	3.62	5.54 ± 0.06^{cB}	2.54	1.08	**
SiO_2-Va	3.04 ± 0.41 ^{e}	4.80	3.90 ± 0.51^{d}	4.90	-0.01	ns
α	***		***			

*Δ reduction: titre reduction (genome copies) between the treatment with 65 mg/mL and that with 20 mg/mL. Different small letters in the same column indicate significant differences in TuV GCs/mL between types of particles, while different capital letters in the same row denote statistically significant differences in TuV GCs/mL between particle concentrations. Significance levels (α): ns (not statistically significant), *(p < 0.05), ** (p < 0.01), *** (p < 0.001).*

Furthermore, the relation between the infectious TuV (Table 3) and the TuV GCs (**Table 4**) for the bare and functionalised particles was analysed. For **SiO_2** at the highest concentration (65 mg/mL), the reduction in the TuV GC (total virus) was slightly greater compared to the infectious virus, which suggests that this reduction was caused by the binding of the virus particles. This relation changed when the SiO_2 particles were functionalised with EOCs. As the reduction in the infectious TuV particles was more marked than for the TuV GCs, this indicates an effect beyond TuV entrapment on the silica particles.

3.4.2. Effect on TuV capsid integrity

One possible mechanism that could explain the observed reduction in the TuV infectious particles is the induction of structural changes to the virus capsid by the functionalised particles. The viral capsid performs crucial functions for virus infectivity, such as attachment to target cell receptors and to protect RNA (Liu et al., 2021). Among the methods for assessing capsid damage is RNase treatment of the sample prior to RNA extraction and RT-qPCR quantification. A reduction in copy numbers indicates a damaged virus capsid, as viral RNA has been degraded by RNase (Li et al., 2012).

Following these principles, RNase treatment was applied before RNA extraction and RT-qPCR for the samples treated with **SiO_2** and **SiO_2-Eu** at 20 mg/mL. As seen in **Table 5**, the difference in the TuV GCs after coming into contact with **SiO_2** was minimal (<1 $\log_{10}$), which indicates a low proportion of virus with severely damaged capsids.

Table 5. The TuV genomic copies (GCs) with and without RNase treatment prior to the RNA extraction of the SiO2- and SiO2-Eu treated virus samples. Mean values ± SD (n = 9).

	Mean $\log_{10}$ $TCID_{50}$/mL			α
Treatment	**Without**	**With RNase**	**Reduction**	
SiO_2	7.47 ± 0.12^{aA}	7.24 ± 0.06^{aB}	0.23	**
SiO_2-Eu	4.04 ± 0.10^{bA}	nd^{bB}	>4.04	***
α	***			

*nd: not detected. Different small letters in the same column indicate significant differences in the TuV GCs/mL between **SiO_2** and **SiO_2-Eu**; different capital letters in the same row denote statistically significant differences in the TuV GCs/mL between w/wo RNase treatment. Significance levels (α): ** ($p < 0.01$), *** ($p < 0.001$).*

In contrast, when samples were treated with **SiO_2-Eu** and RNase, no GCs were observed. This falls in line with the findings of Gilling et al. (2014), who reported severe damage to the MuNoV capsid after treatment with 33.3 mM of carvacrol and RNase.

Lack of detection of the TuV GCs after RNase treatment indicates that the treatment with **SiO_2-EOCs** reduced TuV infectivity through severe damage to the virus capsid.

4. Conclusions

The present study provides novel information about the antiviral effect of EOCs against TuV to be used during drinking water disinfection process. Despite the poor results obtained when employing EOCs in the free form, their immobilisation on silica inorganic supports increased the antiviral effect against TuV. Concretely, treatment of TuV with the functionalised particles reduced the infectious virus with >5 $\log_{10}$ for **SiO_2-Eu** and **SiO_2-Va**, and ca. 4 $\log_{10}$ for **SiO_2-Car** and **SiO_2-Thy**. This study also reveals that treatment with **SiO_2-EOCs** involves the physical retention of TuV to some extent. However, the reduction in TuV infectivity was mostly due to severe capsid damage.

These results suggest that the proposed disinfection process could be used as tertiary or chemical treatment to reduce infectious virus, avoiding further processes, such as centrifugation or sedimentation.

However, additional studies using real water should be conducted to confirm the capacity of **SiO_2-EOCs** to reduce the infectivity of HuNoV or other pathogen viruses in a real environment.

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7. CHAPTER IV
Consumer's perception of using nanotechnology in the agri-food sector

7.1. Nanotechnology in the agri-food sector: Consumer perceptions

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Abstract

The purpose of this work was to evaluate Spanish consumers' opinions on using nanotechnology in food processing and packaging. For this purpose, a literature review was carried out in the main research database to determine the most widespread uses of nanotechnology in the food industry and the most promising developments. Of all the nanotechnology uses in food, five areas of application were identified: developing new ingredients or additives, formulating new antimicrobial systems, and designing new processing methods, sensors and packaging with nanostructured materials. Subsequently, a consumers' opinion study was carried out by means of a survey, in which the opinions and purchase intention of a representative product of all five categories were evaluated, as well as the neophobia level to new food technologies. All the products obtained positive evaluations, and the applications in which nanotechnology did not form part of food were generally better valued than those in which it did form part. The respondents had a medium neophobia level, with an average score of 4.59 (out of 7 points), being consumers with more knowledge about new technologies the least neophobic and those who gave products higher scores. This study provides relevant information for using nanotechnology in the food processing and packaging sector.

Keywords: nanotechnology, food processing, packaging, consumer opinion, neophobia

1. Introduction

The European Commission has reported that nanotechnology is one of the key enabling technologies (KETs) in the Horizon 2020 framework (European Commission, 2020a). The marked revolution that nanotechnology has brought about is because nanomaterials exhibit different functional properties compared to "conventionally sized" equivalents (Royal Society and Royal Academy of Engineering, 2004) given their dimensions. This implies that they have a high surface to mass ratio, which results in higher reactivity. Similarly, the physico-chemical properties of nanomaterials (solubility, shape, etc.) can differ from those of bulk materials, which may entail changes that should be taken into account (Gallocchio et al., 2015).

The food industry has opted for nanotechnology because it is one of the most promising technologies to emerge in recent years. In the food context, Regulation (EU) No. 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods defines engineered nanomaterial as: "any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale". This definition can be subject to changes based on future market developments (European Commission, 2020b).

Besides potential benefits, the use of nanomaterials could present potential risks. On these scales, changes in the above-mentioned properties could pose toxicity problems for humans and animals, as well as environmental damage (Coles and Frewer, 2013). For all these reasons, today nanotechnology is widely studied. Numerous research works have focused on developing different applications in many industrial sectors, as well as on the characterization nanomaterials and toxicity studies.

As this is a new technology, it is very important to know consumer perceptions because these technologies are rejected by most people, such as GMOs (genetically modified organisms), whose potential use in the agri-food field is enormous, but social rejection is a problem to market the products obtained by this technology (Faccio and Guiotto Nai Fovino, 2019; Mohorˇcich and Reese, 2019). In nanotechnology, it is necessary to determine if consumers know this new technology and to know their opinions. It is also important to note that people's perception could differ depending on whether nanotechnology is used as "nanoinside" (the nanomaterial forms part of the product that consumers eat) or "nano-outside" (the nanomaterial is used in processing/packaging, but does not form part of the food product and does not enter the human body).

To date, perception studies related to nanotechnology in the food industry have been carried out in several countries, such as: Australia (Evans et al., 2010), Brazil (Vidigal et al., 2015; Coutinho et al., 2021), Canada (Matin et al., 2012; Roosen et al., 2015), Chile (Schnettler et al., 2013, 2017), Taiwan (Chang et al., 2017) and USA (Zhou and Hu, 2018; Kuang et al., 2020). In Europe, several studies have been carried out in countries such as Austria (Joubert et al., 2020), Germany (Roosen et al., 2015), Italy (Sodano et al., 2016; Viscecchia et al., 2018), Ireland (Handford et al., 2015; Henchion et al., 2019), Switzerland (Siegrist et al., 2007, 2008, 2009) and UK (Gupta et al., 2015; Feindt and Poortvliet, 2020). These studies point out that public perception and knowledge about nanotechnology differ depending on the surveyed region, and that it is also changing over time. Therefore, it is difficult to extrapolate the results from one population to another, and much more, to predict how it will evolve over time.

The aim of this work was to evaluate Spanish consumer perceptions of using nanotechnology in food processing and packaging, in order to predict the acceptation of these technologies in the agri-food sector.

2. Materials and methods

2.1 Preliminary literature review

A review of the main nanotechnology applications under development and those commercialized in the agri-food sector was carried out to select the representative applications of this technology in food products to be included in the consumers' opinion survey. For this purpose, the main research databases (e.g. Scopus, Web of Science, Google Scholar) were consulted by employing the following keywords individually or combined: nanotechnology, nanofood, nanoencapsulation, nanoemulsion, nanoparticles, nanomaterials, nanopackaging, nanosensor, active packaging, intelligent packaging, food sector, food industry, agrifood industry. The "Nanotechnology Products Database", which collects data on distinct nanotechnology products available in different countries (StatNano, 2020), was also consulted. No time restrictions were set.

Of all the different applications found, five products were selected to continue with the second part of the study. This selection was based on the Spanish population's consumer habits, selecting products from different food chain links (production, packaging and quality control), and including both nanotechnology as nano-inside (the nanostructured system is an integral part of food) and nano-outside (those that employ nanotechnology, but it does not form part of ingested food).

2.2 Consumers' opinion study

This part of the study was conducted with an online survey designed in Google-Forms. Call for participants was posted on different social networks (such as LinkedIn, Facebook and WhatsApp) as in other studies (Vuji´c and Szabo, 2022), and the link to the survey was disseminated through these channels. The survey was carried out on a sample of 658 Spanish participants aged over 18, between May and June 2020. Random simple sampling was conducted. The margin of error was below 3.90% for a 95.5% confidence level (k = 2), p = q = 0.5 (principle of maximum indetermination) (**Table 1**).

Table 1. Survey technical specifications.

Target population	Spanish adult people
Sample size	658
Sample error	±3.90
Confidence level	95.5% (k = 2) (p = q = 50)
Sampling procedure	Simple Random Sample
Preliminary questionnaire	Pretest to 20 individuals
Date	May–July 2020

Before the survey began, the participants were informed about the study objective and framework, and that the data provided by them would remain completely anonymous. Then they had to indicate whether they wished to voluntarily participate in the study. The questionnaire was designed as follows:

Part 1 consisted of questions about the participants' demographic and personal data, with two questions on their nanotechnology knowledge and their general opinions of it.

Part 2 included opinion questions on nanotechnology applications in five food products and a question on purchase intention. For this part, first information on nanotechnology and the food industry was provided, as in other similar studies (Siegrist et al., 2008; Roosen et al., 2015; Henchion et al., 2019). Choice of the five food products was based on the criteria defined in Section 2.1. In this way, there were selected two products in which nanotechnology formed part of food (nano-inside) and three products in which it did not (nano-outside).

For each product, the respondents had to indicate their degree of agreement on 7-level Likert scales (the value 1 corresponded to "totally disagree" and the value 7 to "totally agree"). The three items were: "This product seems novel", "This product provides many nutrition or food safety or quality benefits" and "This product poses NO health risk". In addition, they had to establish the extent to which they would like the product and answer a question about their purchase intention. A 5-point scale was used for this last question (the value 1 corresponded to "I would definitively not buy it" and the value 5 to "I would definitively buy it").

Part 3 of the questionnaire consisted of an abbreviated version of the neophobia survey proposed by Cox and Evans (2008), translated into Spanish according to Schnettler et al. (2016), in which consumers had to indicate their degree of agreement with each question on a 7-level Likert scale. A statement on the degree of knowledge about new food technologies was also included (S0).

2.3 Data analyses

Cronbach's alpha was used to test the reliability of the scale of neophobia to new food technologies (questionnaire part 3). With the data obtained in this survey a Kruskal-Wallis analysis was performed for each statement to check if there were any significant differences when considering personal data (gender, level of education or relationship with the agri-food sector). Dunn's procedure with Bonferroni correction was used to test for differences at the 5% significance level. A hierarchical cluster analysis was also carried out to check if there were groups of consumers with different degrees of neophobia. Euclidean distances and Ward's aggregation method were used. Next a Kruskal-Wallis analysis was performed for each question in the neophobia survey by considering the identified clusters.

A Chi-square analysis was carried out to evaluate if there were any differences between clusters in the personal data and other data contained in questionnaire part 1.

Finally, in order to check if there were any significant differences between products in opinions and purchase intention (questionnaire part 2), a Kruskal-Wallis analysis was performed following the same above-described procedure.

The employed statistical program was XLSTAT 2020.3.1 (New York, USA) (Addinsoft, 2020).

3. Results

3.1 Literature review and product selection

After a thorough review of the nanotechnology applications to develop new food products, five application areas were identified. **Table 2** summarizes the main categories and some examples of applications in all these areas. As it can be observed, in the food production field, nanotechnology can be applied to reduce the size of an ingredient or additive, which confers it new properties (solubility, bioavailability, etc.), encapsulate an ingredient or additive, improve its stability or release it in a specific place or situation (gastrointestinal tract, in the presence of microorganisms, etc.). For this category, a low-calorie mayonnaise made by the nanoemulsion technology was chosen as an example of a technology already used in manufacturing food products (Sekhon, 2010). This technology allows the droplet size of an emulsion to become smaller, which enables the use of less fat, while maintaining the original food's palatability. This allows products to be developed with fewer calories than their conventional variants, but without compromising their original organoleptic characteristics.

Another important use of nanotechnology is the design and preparation of new antimicrobials, which are based mostly on natural ingredients. This is the case of nanostructured metals, such as silver, or the nanoencapsulation of compounds with antimicrobial activity to improve the compatibility of the bioactive molecule with food. Indeed propolis, with antimicrobial activity, allows the application in some cases of milder heat treatments (Luis-Villaroya et al., 2015), which can be nanoencapsulated to mask its very strong and undesirable taste. Once encapsulated, propolis can be incorporated into apple juice without compromising its taste, which was the second selected application.

Nanotechnology can also be used as part of the production process without becoming part of food. Nanofiltration, for example, is a less aggressive alternative to conventional processing techniques, such as concentration and clarification of juices or wine dealcoholization (Labanda et al., 2009). Given the novelty of this

process, the third selected product was an alcohol-free wine obtained by nanofiltration to better preserve the final product's organoleptic characteristics.

Other examples in which nanotechnology does not form an integral part of food is found in packaging. Of all the nanotechnology application possibilities in this field, one in which nanotechnology allows the development of active packaging or packaging able to interact with the medium was selected. Specifically, the selected product was apricots (climacteric fruits whose ripening is very fast, which markedly limits their commercial life), which are packed in a film containing a nanomaterial capable of scavenging ethylene, delaying fruit ripening and, thus, prolonging their shelf life (Gaikwad et al., 2020).

Finally, the inclusion of nanosensors in packaging film allows the creation of smart packaging that can detect changes in food or their environment by transmitting this information in the form of different signals (Pérez-Esteve et al., 2013). Some are capable of detecting gases to provide information on packaging integrity. Other systems provide information on food freshness or the accidental freezing of refrigerated food with a colorimetric indicator (Ranjan et al., 2014). A real application of this technology is packaging for meat products, which contains a nanosensor capable of detecting deviations in storage temperature by indicating a break in the cold chain by the irreversible disappearance of barcodes (Enescu et al., 2019).

In summary, nanotechnology can be applied to different food chain links, from food production to packaging and presentation to the final consumer, and depending on its application, in some cases it will form part of the food ("nano-inside" applications) or does not ("nanooutside") (Henchion et al., 2019).

Table 2. Main fields of applications of nanotechnology in the food sector

Goal	Example	Reference
Ingredients or additives		
Improve the bioavailability of some nutrients	High small-sized bioavailable calcium, iron, selenium or coenzyme Q10	Jeon and Lee, 2009; Pereira et al., 2014; Prokisch and Zommara, 2009; Yu et al., 2009
Reduce the dose of an ingredient without compromising its original organoleptic characteristics	Small-sized sodium chloride to be incorporated into biscuits or peanuts; emulsions with a small droplet size that can be incorporated into fat-reduced **mayonnaise** or ice creams, etc.	Moncada et al., 2015; Hamad et al., 2018; Henchion et al., 2019
Modify the physico-chemical characteristics of an ingredient or additive	Small-sized titanium oxide which exhibits high visual transparency with good shielding against ultraviolet light	Latva-Nirva et al., 2009
Protect molecules/microorganisms from processing conditions and the gastrointestinal tract by increasing their solubility, bioavailability, etc., masking sensory characteristics, releasing a cargo to a specific gastrointestinal tract region (targeted release)	Encapsulation of vitamins, probiotics, functional molecules, etc., in nano–/microcapsules	Feher, 2012; Henchion et al., 2019; Mohammadian et al., 2020; Cetinkaya et al., 2021
Antimicrobials systems		
Improve a molecule or substance's antimicrobial power	Silver nanoparticles to be used in the formulation of detergents for washing food, utensils, etc.	Yu et al., 2006; Wilson et al., 2007; Zhang et al., 2009; Mesosilver, 2020; StatNano, 2020
Mask unpleasant sensory characteristics, lower evaporation rates, and improve the compatibility and stability of antimicrobials	Encapsulated antimicrobials, such as essential oils, **propolis**, etc.	Donsì and Ferrari, 2016; Seibert et al., 2019; Tatli Seven et al., 2018

Table 2. Continuation

Goal	Example	Reference
	Processing methods	
Develop processing less aggressive techniques than traditional ones	**Nanofiltration** system for the concentration and clarification of juices or the dealcoholization of beer and wine, etc.	Nath et al., 2018; Peyravi et al., 2020
Provide new supports for enzyme immobilization	Supports for enzyme immobilization	Liu and Dong, 2020; Torabizadeh and Montazeri, 2020
	Packaging	
Create nano-reinforced packaging with improved mechanical or barrier properties	Introduction of nanoparticles (i.e. nanoclays, titanium dioxide) or nanocomposites (nylon resins) into polymeric matrices	StatNano, 2020
Provide food packagers or containers with antimicrobial properties	Introduction of nanometals (i.e. silver, zinc oxide, etc., nanoparticles) into polymeric matrices	StatNano, 2020; Henchion et al., 2019; Pérez-Esteve et al., 2013; Ranjan et al., 2014
Scavenge different compounds (oxygen, ethylene, etc.) from the environment to increase the shelf life of certain foods like fruit, vegetables, meat, etc.	Introduction of nanostructures (i.e. zeolites with potassium permanganate) into polymeric matrices to **avoid fruit ripening**, etc.	Syamsu et al., 2016
	Sensors	
Detect changes in food properties associated with ripening, deterioration, etc.	Nanoparticles of titanium dioxide and methylene blue to indicate the presence of oxygen. Nanosensors that react with volatile compounds in fruit and indicate the degree of ripeness with different colors, etc.	Ranjan et al., 2014; RipeSense®label, 2020

Table 2. Continuation

Goal	Example	Reference
Sensors		
Detect the presence of microorganisms in food	Colorimetric indicators based on noble metal nanoparticles for the detection of foodborne or spoilage microorganisms Colorimetric indicators based on gold nanoparticles to inform about the accidental	Bumbudsanpharoke and Ko, 2019
Detect changes in storage conditions	freezing of refrigerated products. Sensors that indicate if the meat refrigeration temperature has been exceeded by a code on the label disappearing	Ranjan et al., 2014; Enescu et al., 2019

After identifying different nanotechnology application fields in the food industry and selecting the specific applications to be evaluated by consumers, product sheets were created for each selected application (**Figure 1**), on which consumers had to indicate their opinion in the survey.

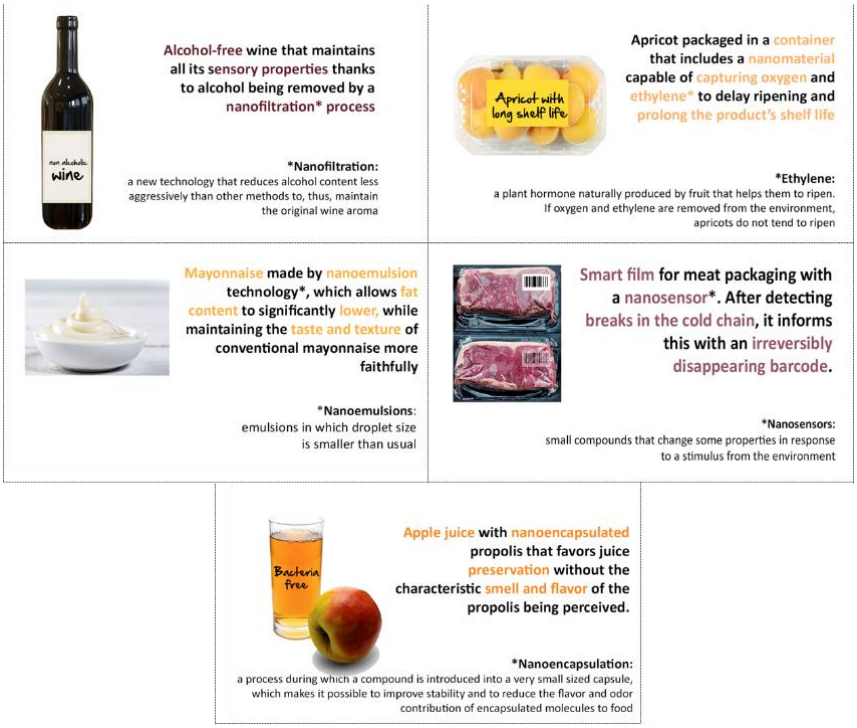

Figure 1. Nanotechnology application in different products that consumers evaluated in the survey.

3.2. Consumers' opinion results

In order to evaluate the opinion of consumers on the use of nanotechnology in food processing and packaging, and their neophobia level as regards new technologies in the food industry, opinion surveys were carried out.

3.2.1. Participants' personal and socio-demographic data

Six hundred and fifty-eight individuals participated in this study. **Table 3** shows their socio-demographic and other personal data. Most respondents were women (64.9%). Their ages ranged from 18 to 79 years, and the age of most participants fell within the 30–49 years range. The majority of people had a high level of education, and 37.5% of participants had some kind of relationship with the agri-food sector. Only 3.5% of the participants indicated following vegetarian/vegan diets, which is a low percentage that would not influence the results. Finally, 15.0% of consumers indicated having an allergy or food intolerance.

3.2.2. Knowledge and general opinion about nanotechnology

Before giving information about nanotechnology and showing the products in which this had been employed, the participants were asked about their knowledge on this new technology and their general opinion. **Table 3** shows their responses. Most participants stated that they knew "something" or "little" (38.8% and 30.4%, respectively) about this technology, while a lower percentage knew "nothing" or "a lot" (16.3% and 12.2%, respectively). Regarding the general opinion on nanotechnology, only three participants had a negative opinion, while 36.2% had a positive opinion and 12.7% a very positive one. The other participants had a neutral attitude ("neither negative nor positive ") and 26.9% were unsure ("I do not know"). No participant perceived this new technology as "very negative".

3.2.3. Neophobia to new food technologies

In order to determine the level of neophobia of the individuals who participated in this study, they completed a survey, as explained in Section 2.2. First of all, they were asked a question about their degree of knowledge on new food technologies, with the statement "I do not know much about new food technologies". The average score given by the respondents was 4.59 (out of 7 points). This value indicates that the participants' average knowledge on new food production technologies did not excel.

Table 3. Participants' personal data and knowledge and opinion about nanotechnology.

		Total		Cluster 1		Cluster 2		Cluster 3		χ^2 (p-value)
		n	%	n	%	n	%	n	%	
		658	**100**	**250**	**38.0**	**228**	**34.7**	**180**	**27.4**	
Gender	Female	427	64.9	164	65.6	143	62.7	120	66.7	ns
	Male	230	35.0	85	34.0	85	37.3	60	33.3	
	Other	1	0.2	1	0.4	0	0	0	0	
Age	18-29	220	33.4	111	44.4	62	27.2	47	26.1	***
	30-49	312	47.4	107	42.8	119	52.2	86	47.8	
	50-65	121	18.4	31	12.4	45	19.7	45	25.0	
	>65	5	0.8	1	0.4	2	0.9	2	1.1	
Educational level	Less than High	34	5.2	12	4.8	8	3.5	14	7.8	ns
	High school	114	17.3	33	13.2	49	21.5	32	17.8	
	Bachelor	510	77.5	205	82.0	171	75.0	134	74.4	
Relationship agri-food sector	Yes	247	37.5	137	54.8	55	24.1	55	30.6	***
	No	411	62.5	113	45.2	173	75.9	125	69.4	
Vegetarians/ Vegans	Yes	23	3.5	5	2.0	10	4.4	8	4.4	ns
	No	635	96.5	245	98.0	218	95.6	172	95.6	
Food Allergic/Food Intolerant	Yes	99	15.0	37	14.8	33	14.5	29	16.1	ns
	No	559	85.0	213	85.2	195	85.5	151	83.9	
Knowledge about nanotechnology	Little	200	30.4	53	21.2	83	36.4	64	35.6	***
	Something	255	38.8	116	46.4	79	34.6	60	33.3	
	A lot	80	12.2	50	20.0	17	7.5	13	7.2	
	I do not know	176	26.9	35	14.0	73	32.0	68	37.8	
Opinion about nanotechnology	Very negative	0	0	0	0	0	0	0	0	***
	Negative	3	0.5	0	0	1	0.4	2	1.1	
	Neither	156	23.8	41	16.4	62	27.2	53	29.4	
	Positive	237	36.2	117	46.8	73	32.0	47	26.1	
	Very positive	83	12.7	56	22.4	18	7.9	9	5.0	

**** $p < 0.001$ (significant differences between clusters); ns: $p > 0.05$ (non-significant differences between cluster.*

Table 4 shows the results of the neophobia survey on using new technologies in food. Some scores were reversed (S5, S6 and S10) so that a higher score for any statement meant a higher neophobia level. Cronbach's alpha of the 10 items was 0.799, indicating good internal reliability. It is important to highlight that this value was 0.843 when the S10 was not included in the analysis, showing an improvement in the reliability of the scale.

A score of 4.0 was obtained as the mean value (minimum and maximum value of 3.3 and 4.7, respectively). Taking into account that a score of 4 is a neutral point, it can be considered that the surveyed population would generally be in an intermediate position in terms of phobia to new food technologies.

Statements were grouped into four factors according to the meaning of the items, in a similar way to the classification proposed by Cox and Evans (2008), with some modifications. The first factor has to do with the perceived usefulness of the technology, the second one with the perceived risk deriving from applying new technologies, the third with new technologies being beneficial given their possibility of offering balanced and healthy diets and better quality food, and the fourth is related to the media's role in providing information (Cox and Evans, 2008). By calculating the mean of each factor, those with lower values were for Factor 1 (utility) and Factor 3 (quality and health), while the risk perception factor obtained a slightly higher score (above 4). The media-related question obtained the highest score, which indicates that the respondents do not trust the information transmitted by the media on new food technologies being balanced and impartial. Although all these questions are based on a validated survey of neophobia to new technologies, it is noteworthy that this last question does not indicate neophobia to new technologies, but refers to people trusting information about new technologies provided by the media. These results agree with the study carried out by Kuang et al. (2020) in USA, except for Factor 4 in relation to the media. They obtained similar values for Factor 1 and Factor 3 (mean scores of 3.38 and 3.13, respectively) and a higher value (4.27) for Factor 2, which agree with our results. However, their lowest value was for Factor 4, while this factor in our study obtained the highest score, which demonstrates consumers' different credibility of the media between both countries.

The Kruskal-Wallis analysis showed that, in general, there were no significant differences ($p > 0.05$) in responses in accordance with gender or level of education. The individuals with a relationship with the agri-food sector exhibited a slightly lower level of neophobia (mean value of 3.7) compared to those not related to the agri-food sector (mean value of 4.1). Age also influenced the responses, with the younger groups generally showing the least neophobia (data not-shown).

Table 4. Mean, standard deviation and median values of the scores given by participants in the survey on neophobia to new food technologies (n = 658).

		Mean	Standard	Media
	Factor 1: New food technologies are unnecessary	**3.8**		
S2	The benefits of new food technologies are often	4.5	1.6	5
S3	There are plenty of tasty foods around, so we do not	3.5	1.9	3
S9	There is no sense trying out high-tech food products	3.5	1.7	3
	Factor 2: Perception of risks	**4.1**		
S5	New food technologies are unlikely to have long	4.0	1.6	4
S7	New food technologies may have long term negative	4.0	1.5	4
S8	It can be risky to switch to new food technologies too	4.3	1.6	4
	Factor 3: Quality and healthy choice	**3.7**		
S1	New foods are not healthier than traditional foods	4.3	1.7	4
S4	New food technologies decrease the natural quality	3.5	1.8	4
S6	New products produced using new food technologies	3.3	1.6	3
	Factor 4: Information / Media	**4.7**		
S1	The media usually provides a balanced and unbiased	4.7	1.6	5

S1-S10: Statements in the order in which they appeared in the survey; (R) The scores in these statements have been reversed, so higher scores indicate more neophobia.

Several authors have studied neophobia to new food technologies in different countries like China (McKenzie et al., 2021), USA (Kuang et al., 2020), Australia (Cox and Evans, 2008; Evans et al., 2010), Canada (Matin et al., 2012), Italy (Verneau et al., 2014), Brazil (Vidigal et al., 2015; Coutinho et al., 2021) or Chile (Schnettler et al., 2017). Based on the results herein obtained, the population of Spain would be more neophobic than the population of China, USA, Brazil or Chile, but less neophobic than those from Australia, Canada and Italy. It should be taken into account that consumer opinions change over time and, with a difference of up to

10 years in some of these studies, the neophobia levels in some of these countries might now be different.

It is also important to note that the standard deviation values were relatively high (**Table 4**), which reflects considerable variability in the participants' responses. To check if there were consumer groups among the respondents with different neophobia levels, a cluster analysis was performed. Three clusters or groups were identified: cluster 1 = 250 individuals, cluster 2 = 228 individuals, cluster 3 = 180 individuals. **Figure 2** shows the mean scores given by each group. The S5 and S6 values were reversed, as explained above. The responses to question S10 are not included in this figure because, as above-mentioned, it does not directly relate to neophobia, rather to the participants' credibility of the media and can distort the graph. The individuals in the three clusters clearly gave different responses, and cluster 1 had the lowest neophobia level (mean of 2.8), while cluster 3 involved the most skepticism toward new food technologies (mean of 5.1). Cluster 2 presented intermediate values (mean of 4.1). The Kruskal-Wallis analysis demonstrated that the differences between clusters were significant in all cases ($p < 0.05$), which indicates segmentation in the surveyed population, with variable neophobia levels (***Table S1***). In the statement about credibility of the media (S10), cluster 1 showed the least confidence in the media. Regarding the participants' knowledge about new food technologies (S0: I do not know a lot about new food technologies), the obtained values were 4.0, 4.9 and 5.0, for cluster 1, 2 and 3, respectively. These findings demonstrated that the respondents with the least neophobia (cluster 1) stated having more knowledge on new food technologies. This indicates that the more information, the less fear or distrust in these techniques.

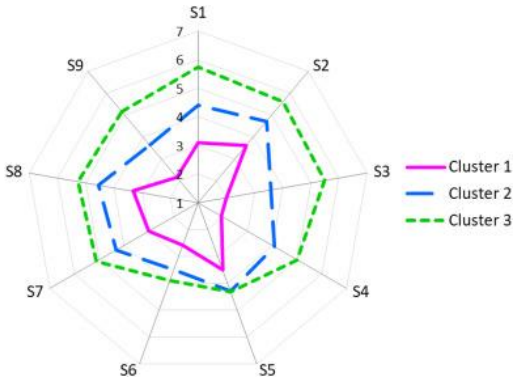

Figure 2. Mean scores given by respondents in the survey of neophobia to new food technology. S1-S9 correspond to the statements shown in **Table 4**. Scores in S5 and S6 have been reversed, so higher scores indicate more neophobia.

In order to check whether there were differences among clusters in terms of socio-demographic and other personal data, and also in relation to knowledge and opinion about nanotechnology (collected in part 1 of the questionnaire), a chi-square analysis was carried out by considering the three clusters. The results are shown in **Table 3**. No significant differences between clusters in terms of gender, level of education, proportion of vegans/vegetarians or allergic/intolerant people were found. The highest proportion of respondents in cluster 1 (less neophobic) were aged between 18 and 29 years old, and 55% stated that they had some kind of relationship with the agri-food sector. The age of the most of the cluster 3 respondents (the most neophobic) ranged from 30 to 49 (similarly to cluster 2) and only 31% stated that they had a relationship with the agri-food sector (**Table 3**). This demonstrates the correlation between age and neophobia, with young people being less neophobic than old people. In a study carried out in China, the authors found that there was a small significant correlation between age and neophobia in the opposite sense because they stated that food technology neophobia decreased with age (McKenzie et al., 2021). This evidence differences between Chinese and Spanish populations.

Table S1. Mean and standard deviation values of the scores given by participants in the survey on the neophobia to new food technology of to the three clusters.

		Cluster 1 (n = 250)	Cluster 2 (n = 228)	Cluster 3 (n = 180)	
S0[(+)]	I do not know a lot about new food technologies	4.0(1.8)[a]	4.9(1.7)[b]	5.0(1.6)[b]	***
S1	New foods are not healthier than traditional foods	3.1(1.6)[a]	4.4(1.3)[b]	5.8(1.1)[c]	***
S2	The benefits of new food technologies are often grossly overstated	3.6(1.6)[a]	4.7(1.2)[b]	5.6(1.1)[c]	***
S3	There are plenty of tasty foods around, so we do not need to use new food technologies to produce more.	2.0(1.1)[a]	3.6(1.3)[b]	5.5(1.2)[c]	***
S4	New food technologies decrease the natural quality of food	2.0(1.0)[a]	4.1(1.2)[b]	5.0(1.5)[c]	***
S5	New food technologies are unlikely to have long term negative health effects (R)	3.5(1.7)[a]	4.3(1.2)[b]	4.3(1.7)[b]	***
S6	New products produced using new food technologies can help people have a balanced diet (R)	2.6(1.4)[a]	3.5(1.3)[b]	3.9(1.7)[b]	***
S7	New food technologies may have long term negative environmental effects	3.0(1.3)[a]	4.3(1.0)[b]	5.1(1.2)[c]	***
S8	It can be risky to switch to new food technologies too quickly	3.3(1.6)[a]	4.6(1.2)[b]	5.3(1.3)[c]	***
S9	There is no sense trying out high-tech food products because the ones I eat are already good enough	2.2(1.0)[a]	3.6(1.2)[b]	5.1(1.3)[c]	***
S10	The media usually provides a balanced and unbiased view of new food technologies (R)	5.1(1.5)[b]	4.6(1.4)[a]	4.4(1.7)[a]	***

[+)]This statement was included at the beginning of the neophobia survey; S1-S10: Statements in the order in which appeared in the survey; (R) Scores in these statements have been reversed, so higher scores indicate greater neophobia
*Different letters in the same row indicate significant differences in the scores between the different clusters; *** p<0.001*

Regarding nanotechnology knowledge in the least neophobic group (cluster 1), most participants (60%) stated that they had some or a lot of nanotechnology knowledge, while the majority in clusters 2 and 3 had little or some nanotechnology knowledge (**Table 3**). Almost 70% of the cluster 1 respondents stated having a positive or very positive opinion of it, while a lower percentage of respondents in clusters 2 and 3 selected the answer "Very positive", and many stated that they had not knowledge. Many also opted for the answer "Neither negative nor positive ".

These results show, therefore, that the younger the Spanish population is, the lower the neophobia level to new technologies. It was also confirmed by the finding that the more acquired information, the lower the neophobia level.

3.2.4. Opinion on nanotechnology applications to different food products

Figure 3 shows the scores given by consumers in the survey on the different products in which nanotechnology has been used. In this part of the study 7-point scales were used, except for purchase intention, which was 5 points, as explained above. However, to better visualize the graph without distortion, the purchase intention score was normalized to a 7-point scale. The higher the score for any question, the better the product valuation. The mean values obtained were higher than 4 points (neutral score) in all cases, which indicates that the respondents had positive opinions of the five food products in which nanotechnology had been employed.

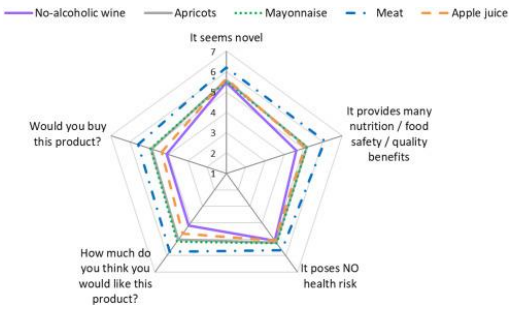

Figure 3. Mean scores given by respondents in the opinion survey on applications of nanotechnology in five food products.

The best valuated product was meat packed in smart film (mean score of 5.9), with significant differences from the other products ($p < 0.05$) for all items. The respondents considered that this product was the newest, that with the most benefits, that with the lowest health risk and that which they would like the most. So they rated it with the highest purchase intention value. This could be due to the fact that nanotechnology in this case only formed part of the packaging and not the product, and was used as an indicator of possible loss of quality, which does not affect product characteristics. This agrees with previous studies in which the perception of products with nanotechnology applied to packaging or "nano-outside" were perceived more positively and purchase intention was higher than when nanotechnology formed part of food products (Siegrist et al., 2007, Siegrist et al., 2008; Roosen et al., 2015; Schnettler et al., 2017; Henchion et al., 2019).

For the questions about product novelty or health risks, no significant differences were found among the other four products. For the other items, the next highest rated products were low-calorie mayonnaise and apricots packed in active packaging, with no significant differences between them for any item (mean score of 5.2 for both products). Although the nanotechnology applied to apricots did not form part of this product, but its packaging, it could have been evaluated worse than meat because this technology does affect normal product evolution as it delays ripening, while nanotechnology had no technological function in meat.

Nanoencapsulated propolis juice (mean score of 5.0) and, especially non alcoholic wine (mean score of 4.7), were the worst rated products, and mainly for the items related to purchase intention and for the question about how much they thought they would like them.

Of the products in which nanotechnology formed an integral part of the product ("nano-inside"), low-calorie mayonnaise was better evaluated than juice with nanoencapsulated propolis, as above-mentioned. The respondents positively evaluated the incorporation of nanotechnology into mayonnaise because it resulted in a healthier product, a characteristic that is highly valued by consumers, as other studies have shown (Henchion et al., 2019).

As the participants in the survey were grouped into three clusters with different neophobia levels, the opinion of products to which nanotechnology was applied was studied per cluster. **Figure 4** shows the scores given by consumers in the survey on the different products, divided into clusters. Cluster 3 with the highest neophobia level scored all the products with the lowest values, while cluster 1 (the least neophobic) gave the highest scores for all the products, which left cluster 2 between both clusters 1 and 3.

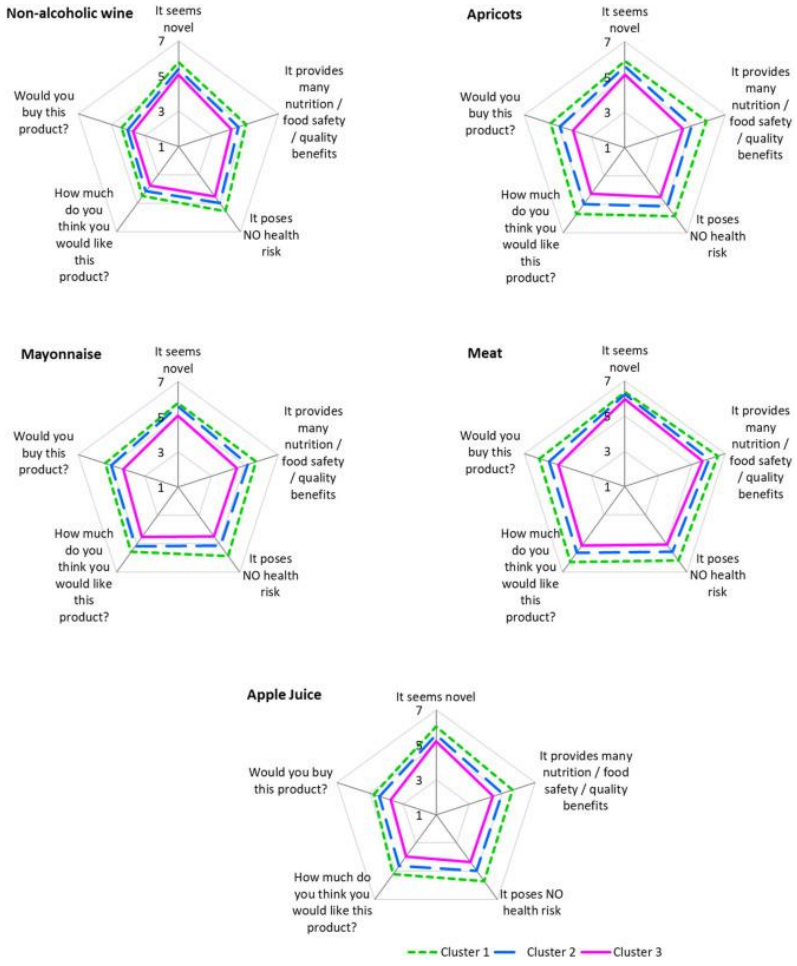

Figure 4. Mean scores given by respondents in the opinion survey on applications of nanotechnology in five food products, considering the three clusters.

These results agree with those of previous studies that used the neophobia scale to new food technologies. Evans et al. (2010) observed how people with high neophobia levels were more likely to reject food produced by new technologies. Matin et al. (2012) correlated neophobia with positions against nanotechnology in food, packaging and any others. In a study carried out in Brazil, Vidigal et al. (2015) observed how the participants with a higher neophobia level less intended to try food produced by new technologies. Sodano et al. (2016) conducted a purchase intention survey and stated that neophobia, among other factors, led to reluctance to buy products with nanotechnology. In another study carried out in Chile by Schnettler et al. (2017) on the purchase intention of different products subjected to new technologies (including nanotechnology), the authors identified a segment with high neophobia who rejected most products, and another with low neophobia levels who gave higher values to purchase intention. These data suggest that information campaigns could reduce the possible rejection of incoporating nanotechnologies into food products. In this sense Kidd et al. (2020) concluded in a study on the use of nanomaterials for in-home drinking water that if manufacturers provided more information about nanomaterial use, as well as the potential benefits an risks, some consumers concerns over these devices could be addressed.

In this study the potential benefits of nanotechnology to the food sector have been shown and that most of the consumers might accept this technology in the formulation, manufacturing, packaging or quality assessment of food products. However, the following limitations of this study should be considered: the sample was relatively homogeneous, especially in terms of age, gender and education, with the majority of participants being young (47.4% were aged 30–49), female (64,9%) and with a high education level (77.5%). Despite this, the Kruskal-Wallis analysis of the socio-demographic data (see Section 3.2.3) showed that no differences were found in the level of neophobia, according to gender or educational level, as did the chi-squared analysis that showed that these factors did not present significant differences between clusters. These findings indicate that gender or educational level in this study were not factors biasing responses. However, age influenced the responses. Although in this work the different perception of the population

according to age has been discussed, future studies should consider citizens in other stages of life. On the other hand, to evaluate the single impact of nanotechnology in the consumer perceptions, a comparison of these products with the same food products without nanotechnology should be carried out in future works.

4. Conclusions

The findings of the present study show that approximately half the Spanish respondents have some knowledge about nanotechnology, and the same percentage values it positively or very positively as only 0.5% had a negative opinion.

The valuation of five different food types to which nanotechnology had been applied to manufacture or package them is generally positive. Moreover, most participants indicate high acceptability and willingness to buy them. Of all the products, packaged meat with nanotechnology incorporated into packaging to inform consumers of the product's quality/safety has been the best valued, which confirms that consumers prefer products in which nanotechnology forms no part of food (nano-outside).

The good product acceptability falls in line with the observed low neophobia level. In general, the Spanish respondents have an intermediate neophobia level. However, there are segments with significantly different neophobia. The younger age groups and groups with more knowledge about new technologies exhibit less neophobia. As the most informed have been those who best value the use of nanotechnology, information campaigns carried out mainly by sector organizations or official bodies could reduce the possible rejection of incorporating nanotechnologies into food products.

This study shows that, unlike other technologies, the use of nanotechnology in the food field would not negatively impact the food product image for the surveyed Spanish population. This is a good boost for food industries interested in implementing nanotechnologies into their processes and products, although future studies should be performed to include a broader range of products as well as a

greater participation of older people, who may have a higher level of neophobia and, therefore, a lower level of acceptation of products with nanotechnology.

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8. GENERAL DISCUSSION

In the introduction of the present doctoral thesis, it has been shown that the use of purified natural antimicrobials derived from plants, such as essential oil components (EOCs), is effective in controlling a broad spectrum of microorganisms, including bacteria, fungi, and yeasts, in both *in vitro* and *in vivo* environments. However, their direct application to food matrices is a challenge due to their low solubility in aqueous media and their ability to alter the color, taste and odor of the food products to which they are added. In response to these limitations, and in line with the increasing consumer preferences for natural ingredients, alternative approaches to the direct application are being explored. Among those, one of the most promising technology involves the immobilization of EOCs onto inorganic supports.

Considering that chemical immobilization is a complex process aimed at preserving the antimicrobial properties of a target molecule on the surface of the carrier, it has been stated that the specific properties of both the silica surface (composition, charge, hydrophilicity/hydrophobicity, chemical stability, roughness and geometry) and the EOCs (size, charge or molecular structure) allow covalent binding. In addition, the immobilized form of EOCs onto silicon oxide particles have shown to be non-toxic to different cell lines, thus making its application in food preservation promising.

Having this in mind, different authors have assessed the antimicrobial activity of the aforementioned immobilized EOCs against different microorganisms, both *in vitro* and *in vivo*. In those studies, it has been identified two different approaches for their application. On the one hand, immobilized EOCs could be as additives, being presented in the final product. On the other hand, the same materials could be used as processing aids, not being part of the food (i.e., filtration aids). The first approach has proven to be effective in jam, whereas the latter has shown promising for the microbial stabilization of apple juice, craft beer, or white wine after a filtering process through the particles. However, the addition of the developed antimicrobials to milk as additives or the filtration milk or tiger nut-derived beverages through the particles resulted ineffective.

Due to the challenges with directly applying EOCs, along with the lack of understanding about why antimicrobial activity is sometimes lost in some food products, using immobilized particles for food preservation reveal some research gaps. In addition, there are no studies evaluating the effect of these antimicrobials against spoilage microorganisms and viruses. These gaps, together with the study of nanotechnology consumer acceptance, are the main focus of this thesis. Having this in mind, the present thesis has evaluated how different food components naturally present in food (proteins, lipids, carbohydrates, organic acids, and ethyl alcohol) modify the antimicrobial activity of free essential oil components (carvacrol, eugenol, geraniol, thymol, and vanillin). Secondly, the effect of the above-mentioned food components on the antimicrobial activity of vanillin immobilized on silicon oxide particles has been determined. After assessing the effect of EOCs-food matrices interaction on antimicrobial activity of the EOCs, the next step involved the validation of this approach through *in vivo* applications. For that, different EOCs (eugenol, geraniol, thymol and vanillin) were employed against a spoilage microorganism, *Alyciclobacillus acidoterrestris* in orange juice. In parallel, the antiviral activity of carvacrol, eugenol, thymol and vanillin against Tulane virus was evaluated in an aqueous medium. Finally, consumer perceptions about the use of nanotechnological applications in food products were studied.

By addressing these research gaps, the present thesis aims to provide valuable information on the applicability and effectiveness of immobilized EOCs on the quality and safety of liquid foods. Structuring the thesis into four chapters and five scientific articles, this thesis provides a comprehensive framework to address the main objectives in a systematic way. Having this in mind, this section aims to sequence and discuss the results obtained in an integrated manner.

Chapter I aims to evaluate the effect of interactions between various EOCs and naturally occurring components (i.e., proteins, lipids, carbohydrates, organic acids and alcohols) present in food matrices against *E. coli*, a bacterium commonly used as a reference. In the first article of this chapter, **Article 1**, the antimicrobial activity of five EOCs (carvacrol, eugenol, geraniol, thymol or vanillin) has been evaluated in the presence of bovine serum albumin (BSA) (proteins), sunflower oil (lipids), D-lactose, D-sucrose, pectin and starch (carbohydrates), citric acid (organic acids) and

ethanol (alcohol) at a concentration that covers the main food liquid products. For that, different time-kill curves of the microorganism have been built.

In this article, the evaluation was first carried out with vanillin, as it is the most widely used EOC in food technology. Results revealed that the effects of BSA, sunflower oil and D-lactose were especially noteworthy on the vanillin antimicrobial activity, followed by D-sucrose and starch. In the presence of these components, vanillin lost most of its antimicrobial activity. Based on the results obtained, the antimicrobial activity of other phenols such as Car, Eu or Thy, or alcohols such as Ger, was evaluated in presence of the three food components that most inhibited the antimicrobial activity of Va. Results of the second part of the study showed that the behavior of the mentioned EOCs was completely different from that reported for vanillin, keeping totally or partially their antimicrobial activity. Analyzing the results individually, geraniol was the only compound that maintained its antimicrobial activity after contact with protein-containing media, being thus the most appropriate antimicrobial for protein-rich media. This could be due to the fact that geraniol has neither aldehyde nor phenol in its structure, groups that would be blocked by its tendency to form adducts with proteins. In sunflower oil-containing media, eugenol was the antimicrobial that best kept its antimicrobial activity, probably due to a lower affinity for lipid systems than the other EOCs. In D-lactose-containing media, carvacrol, eugenol, geraniol, or thymol kept effectiveness in reducing bacteria, probably due to a lower interaction with the food component compared to vanillin. In a subsequent step, an analysis of the effect of food components on the EOC concentration was carried out to assess if differences in antimicrobial activity could be attributed to the EOC concentration after interacting with food components. The findings confirmed that vanillin would lose its activity due to the adduct formed with the protein as the interaction did not result in a noticeable loss of EOCs effective concentration. On the contrary, in the case of sunflower oil, the loss of antimicrobial activity of Car and Thy could be due to the lower EOC concentration available to react. Finally, the interaction of EOCs with D-lactose did not significantly reduce the effective concentration, indicating another type of interaction beyond those studied.

After studying the effect of free EOCs-food component interactions, **Article 2** determined the antimicrobial activity of immobilized vanillin on silica oxide particles after interaction with the above-mentioned food components. For this purpose, immobilized vanillin was used as processing aid, concretely as filtering material. Results showed that the food components similar to those investigated in the previous article, i.e. BSA, sunflower oil and D-lactose, starch and pectin (with the exception of D-sucrose) inhibited the activity of the system. On the contrary, the citric acid and ethanol improved the vanillin antimicrobial activity. In this study, dual combinations of food components that hindered antimicrobial activity and those that enhanced antimicrobial activity (citric acid or ethanol) were also evaluated. In this sense, results showed that the combination provoked an increase of antimicrobial activity when compared with the individual hindering food components, overcoming in some cases their blocking effect. This was also evidenced in previous works, where high effectiveness of the systems was only observed in matrices containing acids (apple juice) or alcohol (beer and white wine).

In summary, the correlation of results obtained in Articles 1 and 2 (**Chapter 1**) together with those previously reported in real food matrices (i.e., apple juice, beer, tiger nut-derived beverage or milk) confirmed the importance of analyzing the food matrix to select the most suitable antimicrobial system. Considering the results of **Chapter 1**, orange juices and water have been selected as *in vivo* media for studying the antimicrobial activity of the free and immobilized EOCs.

Chapter 2 opens new uses for functionalized particles by exploring their antimicrobial effect on an emerging spoilage microorganism, *Alyciclobacillus acidoterrestris* in orange juice. **Article 3** evaluated the effect of different EOCs on bacterial growth and on the inhibition of guaiacol production, the main molecule generated by *A. acidoterrestris* that causes an organoleptic alteration of juices. This emerging microorganism presents a significant challenge to the juice industry due to its exceptional thermal resistance. In this research, it was observed that the most active essential oil components in *in vitro* tests were eugenol and thymol, followed by vanillin and geraniol. Considering the above-mentioned results, eugenol and thymol were selected to carry out the *in vivo* assays in orange juice. In addition, the immobilized antimicrobials were tested as additives or processing aids. In the first

approach, immobilized forms of both, eugenol and thymol, maintained their activity compared to the application in their free form for 24 h. Concretely, the use of the minimum inhibitory concentration (MIC) in *in vitro* assays reduced ca. 3 $\log_{10}$ the growth of bacteria and inhibited the production of guaiacol to undetectable levels in orange juice. This fact is of great interest since immobilization would overcome the organoleptic alterations of the product derived from the direct application of free EOCs. On the other hand, when using the immobilized EOCs as filtering materials (processing aids), a complete inhibition of guaiacol production by the microorganism was found. Given the significance of secondary metabolites in generating off-flavors and odors that might lead to consumer rejection and economic losses, both approaches could serve as an alternative treatment in the juice industry. In addition, the efficacy of the system agrees with the predictions made in the previous articles related to the effect of the food matrix.

Parallel to the evaluation of immobilized EOCs against a spoilage microorganism, the subsequent chapter of this thesis aimes to investigate their efficacy against viruses (**Chapter 3**). **Article 4** studied the effect of carvacrol, eugenol, thymol, and vanillin, both in their free and immobilized form, on Tulane virus, in aqueous medium. Multiple independent assays were first conducted to quantify infectious virus particles within rhesus monkey kidney epithelial cells (LLC-MK2 cells). Following this assay, total genomic copies after EOC treatments were determined by RT-qPCR and possible damage to the virus capsid was analyzed by RNase pretreatment prior to RT-qPCR. The results have shown that immobilization of EOCs remarkably increased their antiviral activity. Specifically, eugenol and vanillin achieved a reduction of >5 $\log_{10}$ in infectious particles, while carvacrol and thymol achieved a reduction of approximately 4 $\log_{10}$. Under the same conditions but using free EOC, a reduction of about 1.4 $\log_{10}$ was achieved. Regarding the quantification of total viral particles, the immobilized EOCs reduced the number of viral particles. However, the greater reduction in infectivity compared to the total number of virus particles could indicate an antiviral effect beyond virus entrapment in the particle. In this sense, an assessment of capsid damage confirmed that functionalized particles were able to modify or degrade the virus capsid, the part of the virus responsible for its infectious properties. These findings reveal, for the first time, the

antiviral potential of functionalized particles, thus extending their utility beyond the reported use against bacteria, fungi or yeast.

Despite the proven efficacy of the use of EOCs immobilized on silicon oxide particles against bacteria and viruses, their practical application in the food industry presents several challenges. Among them, it is important whether these technologies would be accepted by consumer (**Chapter 4**). Concretely, **Article 5,** studied consumers' knowledge and perception of using nanotechnology in food processing in order to predict the acceptation of these technologies in the food sector. For this purpose, a survey was designed. A total of 658 Spanish people participated in the study. The results indicated different levels of familiarity with nanotechnology, with 16.3% and 12.2% reporting "nothing" or "a lot" of knowledge, respectively, while the majority claimed "something" or "little" understanding. Regarding neophobia evaluation, the results revealed an intermediate position. However, respondents showed a slightly higher perception of the risk associated when technology is incorporated into products. Cluster analysis identified three distinct respondent segments. The different correlations between the survey statements suggests that the more information, the less apprehension or distrust towards these techniques. Furthermore, younger participants displayed lower levels of neophobia compared to older participants, probably due to their greater familiarity with technology. In addition, the study underlined that participants who are more informed about nanotechnology tend to have a more positive opinion. Consequently, promotion initiatives could play a crucial role in mitigating potential resistance to the integration of nanotechnology into food production. In terms of the products evaluated, nanotechnology application as processing aids, which are removed during food processing (nano-outside), received more favorable views compared to when the technology was applied as additives, which remain in the food (nano-inside).

In conclusion, this thesis underscores the potential of immobilizing essential oil components on silicon oxide supports as viable alternative techniques, either as substitutes or complements to conventional treatments like pasteurization, sterilization, and chlorination. Through comprehensive investigation, this doctoral thesis not only clarifies that the efficacy of the natural antimicrobials compounds is

strongly influenced by the food matrix, but it also identifies the most suitable essential oil component that should be applied on a specific medium. In addition, this doctoral thesis shows for the first time the efficacy of EOCs immobilized on silicon oxide particles, used as additives or processing aids, not only to control the growth of spoilage microorganisms, but also to inhibit the production of metabolites that cause product spoilage, food waste and economical losses. Additionally, the use of immobilized EOCs have resulted effective against viruses. Finally, the research indicated that nanotechnological applications used as processing aids tend to be perceived more positively by consumers than those applied as components that remain in the food. These findings are very interesting when validating the different treatments that have been carried out throughout this doctoral thesis. For instance, the use of antimicrobials as processing aids to guarantee the quality of orange juice or to reduce infective virus particles in aqueous medium could be viewed favorably by consumers. Thus, since it is a very promising technology, both research and dissemination efforts should be made to ensure that more and more consumers perceive the application of each of the proposed technologies positively.

9. CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions and future perspectives

1. Immobilizing essential oil components on silicon oxide particles offers a promising food preservation technology. Concretely, the immobilized EOCs antimicrobial activity is maintained or enhanced without altering food properties. They do not show cell toxicity and are effective against bacteria, fungi, and yeast, in both *in vitro* and *in vivo* studies on fruit-derived foods.

2. Matrix composition, in general, negatively influences EOCs activity through diverse interactions between food components and antimicrobials, especially in media containing proteins, lipids and carbohydrates. The antimicrobial hindering depends on the EOC type. In media containing bovine serum albumin (proteins), geraniol has been shown to be effective, unlike other EOCs. In contrast, eugenol has been confirmed to be the most effective in media containing sunflower oil (lipids), and carvacrol, eugenol, geraniol or thymol would be recommended in case of media containing D-lactose (carbohydrates).

3. The antimicrobial activity of vanillin immobilized on silicon oxide particles is inhibited in the presence of the same food components that in the free form (proteins, lipids and some carbohydrates). However, some combinations of inhibitory food components with those that produce an increase in activity (acid or ethanol) could make the system effective.

4. Eugenol and thymol immobilized onto silicon oxide particles have been proved effective against different strains of a spoilage thermoresistant microorganism such as *Alyciclobacillus acidoterrestris* in *in vivo* experiences with orange juices. In addition, both antimicrobials have inhibited guaiacol formation by the bacteria to undetectable levels. Thus, food-antimicrobial contact for a certain period of time or filtration of orange juice through a bed of particles functionalized with eugenol or thymol could provide a solution to one of the main challenges in the juice industry, avoiding both, heat treatments and organoleptic alterations of the product by spoilage microorganisms.

5. Immobilization of carvacrol, eugenol, thymol and vanillin on silica microparticles has the potential to reduce human norovirus infectivity to undetectable levels, being the reduction in TuV infectivity mostly due to a severe capsid damage. As the particles have not exhibited any potential cytotoxicity and the treatment avoids the using of chlorination, this novel treatment could meet the strict disinfection requirements for public water system without presenting any potential toxicological risk.

6. Consumers' acceptance of different food submitted to preservation methods based on nanotechnology has been proved to be high. Consumers prefer products in which nanotechnology forms no part of food. Based on these findings, it would be more convenient to use the developed silicon oxide particles functionalized with EOCs as processing aids rather than as food preservatives.

7. It can be concluded that the covalent immobilization of essential oil components on food grade supports to be used in the food industry as food preservatives or processing aids is feasible, promising, ecofriendly and a well-accepted strategy to fight against traditional pathogens, as well as emerging bacteria and viruses. However, to optimize the efficiency of these antimicrobial systems, not only the type of target microorganism must be taken into account (traditional approach), but also the composition of the food matrix.

8. Future works should investigate the effects of other natural food components, such as different types of proteins or lipids, on the EOCs antimicrobial efficacy. Furthermore, to improve the understanding of this novel technology, studies should be carried out on the interaction of antimicrobial-food components and new families of antimicrobials, as well as studies on other altering or pathogenic microorganisms.

10. SCIENTIFIC CONTRIBUTIONS

List of scientific articles

Gómez-Llorente, H., Hervás, P., Pérez-Esteve, É., Barat, J. M., & Fernández-Segovia, I. (2022). Nanotechnology in the agri-food sector: Consumer perceptions. *NanoImpact, 26,* 100399. https://doi.org/10.1016/j.impact.2022.100399 Impact factor 4.9 and Cite score 8.9.

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Poster communications

Karakas, E; Pérez-Esteve, É; **Gómez-Llorente, H**; Rivas-Soler, A; Fernández Segovia, I; Barat Baviera, J.M. (2021). Effect of water hardness and dirt on the antimicrobial effectiveness of silica microparticles functionalized with vanillin. XIV *International Workshop on Sensors and Molecular Recognition* (IWOSMOR 2021). Valencia, España: Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico.

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Predoctoral stay in a foreign institution

Predoctoral stay in Virology group from Norwegian University of Life Sciences (Ås, Norway). From September to November of 2021. Learning to analyze the infectivity of different viruses employing animal cells and to quantify virus particles by RT-qPCR.