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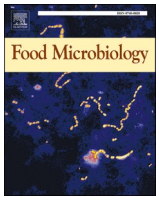

Antimicrobial activity of essential oil components against *Escherichia coli* depends on the food components present in a food matrix

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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.

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, 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 enterica*, 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 (Fig. S1). 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)

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(Fig. S1). 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 (Fig. S1). 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 (Fig. S1). 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 (Fig. S1). 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).

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) determined 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) examined 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 revealed 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) reported 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) indicated 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) showed 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) reported 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 showed 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. Material 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 following the CECT instructions by two consecutive seeding in PCA at 37 °C at 24 h intervals. A differentiated colony was then transferred to 10 mL of TSB and incubated at 37 °C for 24 h. PCA and TSB were prepared following the manufacturer's instructions. The cell concentration of the inoculum was 9 $\log_{10}$ CFU/mL. This concentration was determined by measuring the optical density employing a Helios Zeta UV–VIS device (Thermo Scientific, Hampton, New Hampshire, USA) at a wavelength of 600 nm and it was verified by determining bacteria concentration in the inoculated food components using the plate count method (Gómez-Llorente et al., 2024).

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.

Phosphate Buffered Saline solution was 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 and adjusted to 7.4 using 1 M HCl (Sahakijpijarn et al., 2019). Once PBS solution was prepared, each food component was dissolved in the solution using an Ultra Turrax® homogenizer (T-25,

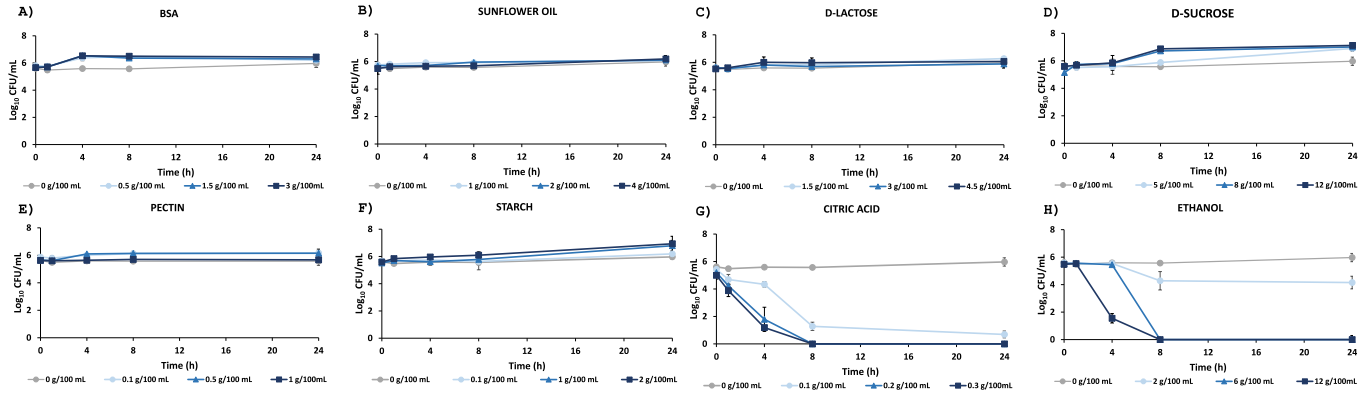

Fig. 1. Time-kill curves for *E. coli* treated with bovine serum albumin (BSA) (A), sunflower oil (B), D-lactose (C), D-sucrose (D), pectin (E), starch (F), citric acid (G), and ethanol (H) in absence of essential oil components.

IKA, Germany) at a speed of 9500 rpm during 5 min (Cabrera-Trujillo et al., 2018).

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 of EOCs against *E. 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 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 mL), sunflower oil (4 g/100 mL) or lactose (4.5 g/100 mL) 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 × 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. Fig. 1 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.

Table 1
Minimum Inhibitory Concentration (MIC) of eugenol (Eu), geraniol (Ger), thymol (Thy) and vanillin (Va) against *Escherichia coli* K12. The results are expressed as mg/mL. Mean value (n = 3).

	MIC
Eu	0.6
Car	0.2
Ger	0.8
Thy	0.2
Va	2

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 (2 mg/mL) in Table 1. This value 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) (Fig. 2). The results are discussed below.

3.2.1. BSA and sunflower oil

As shown in Fig. 2A, 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. This might be due to the formation of BSA-Va adducts (Bashiri et al., 2021).

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) ($p < 0.001$) (Fig. 2B). 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. Regarding the mechanism of blocking the antimicrobial activity of Va by sunflower oil, some authors hypothesized that this food component would create a layer that would partially reduce the antimicrobial contact with bacteria (Cui et al., 2016; Gómez-Llorente et al., 2024).

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 (Fig. 2C). 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 (Fig. 2D). 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 ($p < 0.05$) (Fig. 2E). 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 capacity of pectin to form hydrogels. According to Yang et al. (2020) the enhancement of antimicrobial activity would be mainly due to two different reasons. On the one hand, the hydrogels would increase the disruption of bacterial membrane by changing the membrane potential. On the other hand, the hydrogels formation would improve the stability and solubility of hydrophobic free antimicrobial molecules in physiological environments, which could facilitate the internalisation of this antimicrobial compound into the bacterium. 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 ($p < 0.001$) (Fig. 2F). 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

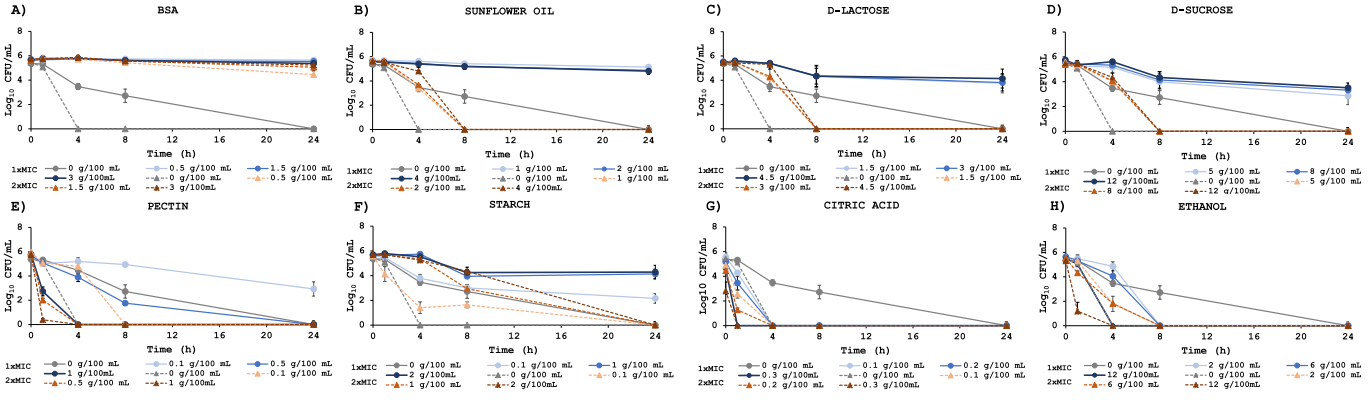

Fig. 2. Time -kill curves for *E. coli* treated with MIC × 1 (continuous blue lines) and MIC × 2 (dashed orange lines) concentrations of vanillin (Va) and in presence and in the absence of diverse concentrations of bovine serum albumin (BSA) (A), sunflower oil (B), D-lactose (C), D-sucrose (D), pectin (E), starch (F), citric acid (G) and ethanol (H). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

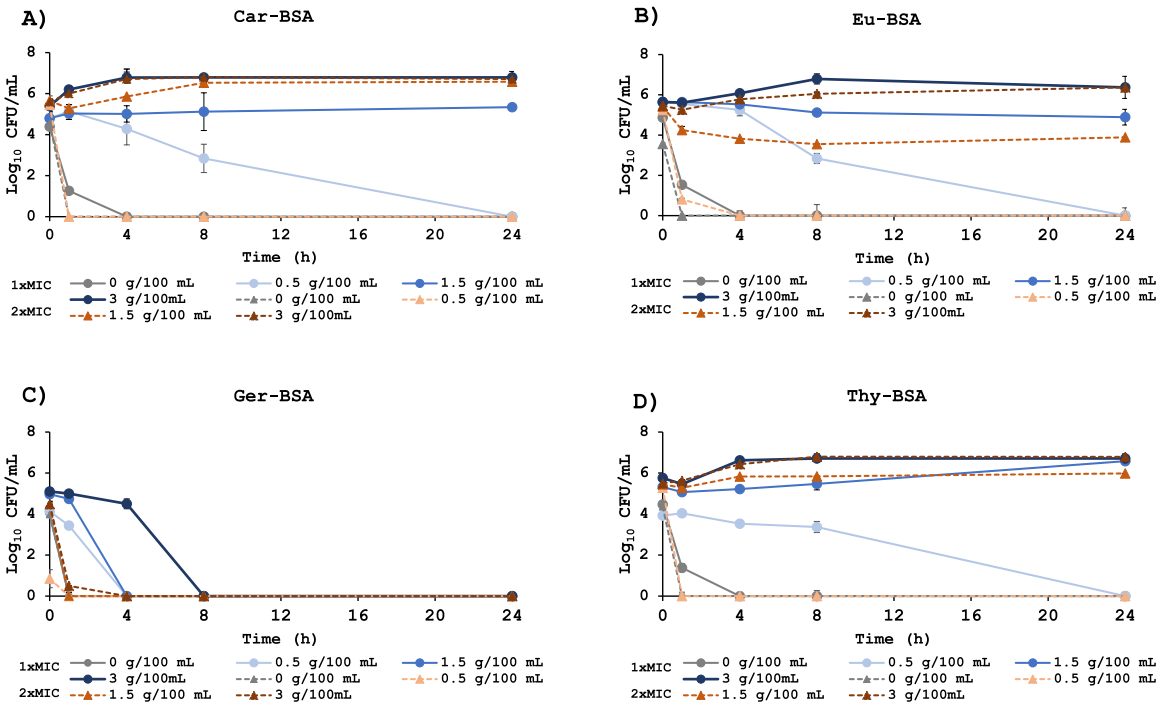

Fig. 3. Time-kill curves for *E. coli* treated with MIC × 1 (continuous blue lines) and MIC × 2 (dashed orange lines) concentrations of carvacrol (Car) (A), eugenol (Eu) (B), geraniol (Ger) (C) and thymol (Thy) (D), in presence and in the absence of diverse concentrations of bovine serum albumin (BSA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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 (Fig. 2G and H).

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 (Fig. 2H).

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).

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 reveal 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,

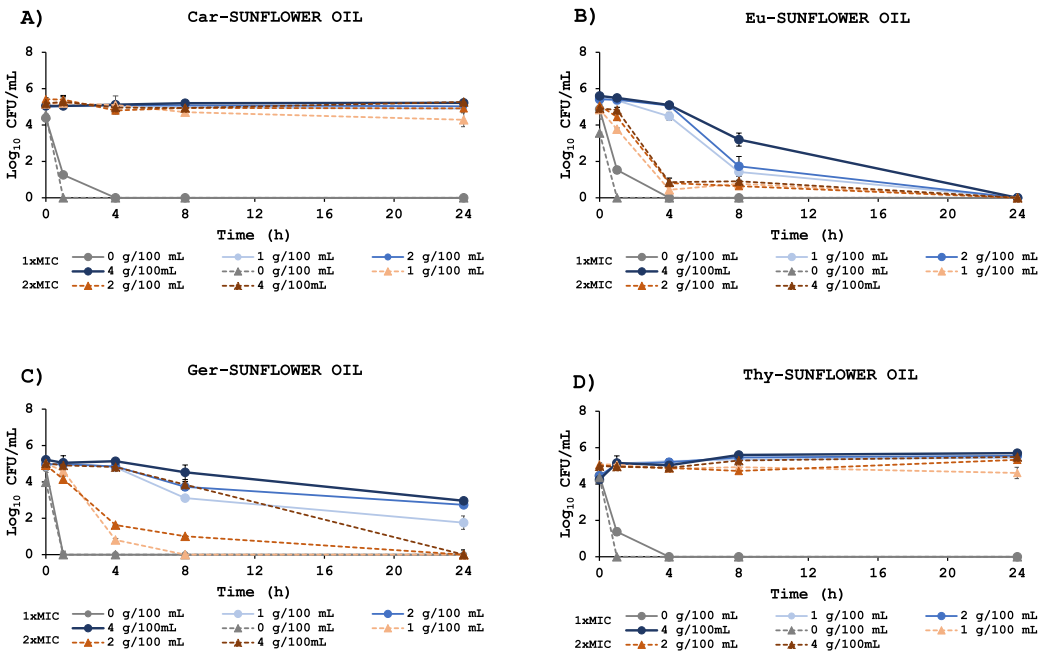

Fig. 4. Time-kill curves for *E. coli* treated with MIC × 1 (continuous blue lines) and MIC × 2 (dashed orange lines) concentrations of carvacrol (Car) (A), eugenol (Eu) (B), geraniol (Ger) (C) and thymol (Thy) (D), in presence and in the absence of diverse concentrations of sunflower oil. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and indicates an interactive effect among these variables.

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 (0.2 mg/mL), Eu (0.6 mg/mL), Thy (0.2 mg/mL), and Ger (0.8 mg/L) against *E. coli* were firstly determined (Table 1). 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

Fig. 3 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/100 mL 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 would interact more weakly with the functional groups responsible for antimicrobial properties. 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).

3.3.2. Sunflower oil

Fig. 4 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 and 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

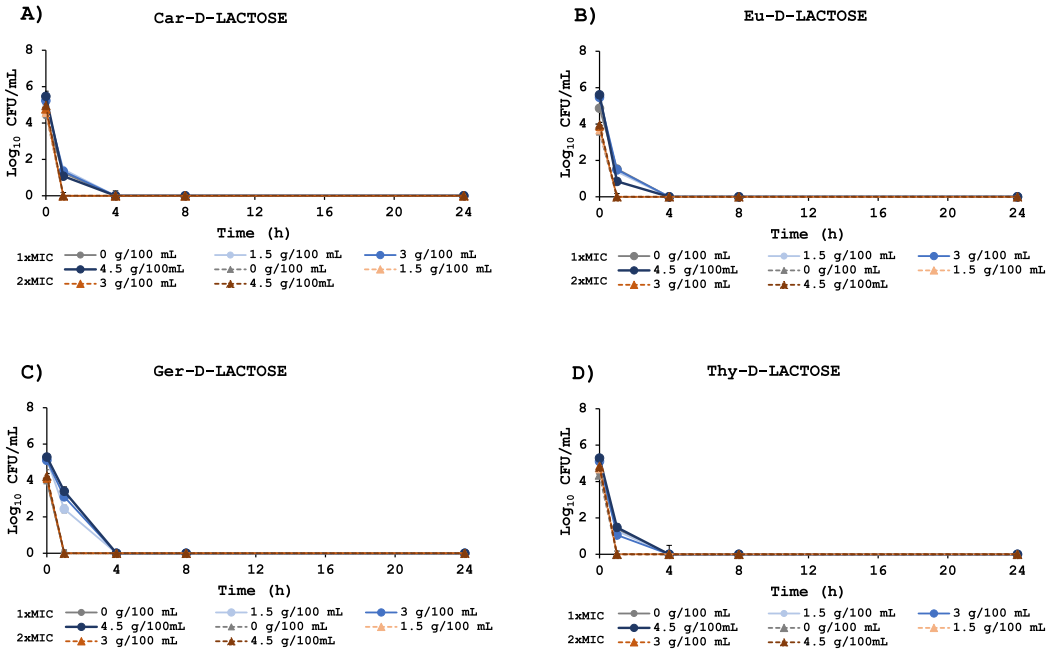

Fig. 5. Time-kill curves for *E. coli* treated with MIC × 1 (continuous blue lines) and MIC × 2 (dashed orange lines) concentrations of carvacrol (Car) (A), eugenol (Eu) (B), geraniol (Ger) (C) and thymol (Thy) (D), in presence and in the absence of diverse concentrations of D-lactose. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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.

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

Table 2
Free concentration (mg/mL) of Car, Eu, Ger, Thy and Va in the presence and in the absence of BSA, sunflower oil and D-lactose in the growth media at different incubation times.

Food constituent	EOC	Concentration of food constituent (g/100 mL)	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
D-lactose	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
	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

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

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 Fig. 5. 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.

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 Fig. 5 clearly evidenced.

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 2 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 2). 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 (Fig. S1). 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 S1). 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.

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

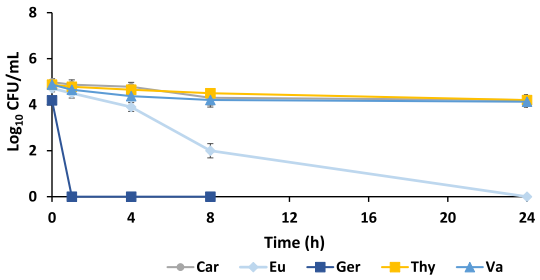

Fig. 6. Time-kill curves for *E. coli* treated with carvacrol (Car) (0.04 mg/mL), eugenol (Eu) (0.46 mg/mL), geraniol (Ger) (0.65 mg/mL), thymol (Thy) (0.03 mg/mL) and vanillin (Va) (0.62 mg/mL).

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 (Fig. 6). 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.

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 revealed that BSA, sunflower oil and some carbohydrates inhibit EOCs antimicrobial activity. Furthermore, the 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 other microorganisms like gram-positive bacteria, 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.

CRediT authorship contribution statement

Héctor Gómez-Llorente: Writing – original draft, Methodology, Formal analysis, Data curation. **Édgar Pérez-Esteve:** Writing – original draft, Supervision, Methodology, Data curation, Conceptualization. **José M. Barat:** Funding acquisition, Conceptualization. **M. Consuelo Jiménez:** Writing – review & editing, Formal analysis. **Concepción González-Bello:** Writing – review & editing, Formal analysis. **Isabel Fernández-Segovia:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2024.104638.

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