UNIVERSITAT POLITÈCNICA DE VALÈNCIA

MATRIX EFFECT ON THE EFFICACY OF VANILLIN-COATED SUPPORTS FOR THE MICROBIAL STABILIZATION OF DRINKS

FINAL PROJECT OF MASTER'S DEGREE IN FOOD SCIENCE AND ENGINEERING

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ABSTRACT

Food industry is in urge to develop new technologies to mitigate or even reduce the negative effects of conventional conservation methods (such as thermal processes or the use of chemical preservatives). One of the recently proposed strategy to reduce the microbial load of different liquid food products involves the use of silica particles functionalized with vanillin as a filtering bed. However, it has been noticed that the antimicrobial power of these particles changes depending on the food matrix (juice, milk, wine…). This study is focused on clarifying the effect of single constituents of typical food matrices (salts, proteins, carbohydrates, lipids, ethanol, acids) on the capability of the filtration system to reduce or eliminate the initial microbial charge. To this end, silica micro particles (50-110 µm) were functionalized with vanillin. Different food models with different concentrations of bovine serum albumin, sucrose and lactose, sunflower oil, citric acid, ethanol, $MgCl_2$, $CaCl_2$ and $NaHCO_3$ were prepared. For each concentration, 100 mL of inoculated food models with *Escherichia coli* were filtered through a layer of vanillin-functionalized supports to assess the initial removal capability. First, the maximum concentration of the different organic and inorganic compounds that can be used until the filter lose its activity was determined. Then, a shelf-life study defined as the number of liters that can be filtered until losing the reduction capacity of three logarithmic cycles was carried out. Results showed that while the inorganic constituents do not affect the antimicrobial capacity, the presence of organic compounds on the filtered media can have different impacts on the filter's antimicrobial effectivity. While proteins, lipids or lactose reduce the shelf-life up to a 11%, the presence of organic acids or alcohol increases it close to a 200%.

KEYWORDS: Silicon oxide, natural antimicrobial, filtration, stabilization

RESUMEN

La industria alimentaria urge desarrollar nuevas tecnologías para mitigar o incluso reducir los efectos negativos de los métodos de conservación convencionales (como los procesos térmicos o el uso de conservantes químicos). Una de las estrategias propuestas recientemente para reducir la carga microbiana de diferentes productos alimenticios líquidos consiste en usar de partículas de sílice funcionalizadas con vainillina a modo de lecho de filtración. Sin embargo, se ha observado que el poder antimicrobiano de estas partículas cambia en función de la matriz alimentaria (zumo, leche, vino…). Este estudio se centra en aclarar el efecto de los componentes individuales de las matrices alimentarias típicas (sales, proteínas, carbohidratos, lípidos, etanol, ácidos) sobre la capacidad del sistema de filtración para reducir o eliminar la carga microbiana inicial. Para ello, se funcionalizaron micropartículas de sílice (50-110 µm) con vainillina. Se prepararon diferentes modelos de alimentos con diferentes concentraciones de albúmina sérica bovina, sacarosa y lactosa, aceite de girasol, ácido cítrico etanol, $MgCl_2$, $CaCl_2$ y $NaHCO_3$. Para cada concentración, se filtraron 100 mL de modelos de alimentos inoculados con *Escherichia coli* a través de una capa de soportes funcionalizados con vainillina. Primero, se determinó la concentración máxima de los diferentes compuestos orgánicos e inorgánicos que se pueden utilizar hasta que el filtro pierde su actividad. Luego, se realizó un estudio de vida útil, definido como la cantidad de litros que se pueden filtrar hasta perder la capacidad de reducción de tres ciclos logarítmicos. Los resultados mostraron que, si bien los componentes inorgánicos no afectan la capacidad antimicrobiana, la presencia de compuestos orgánicos en el medio filtrado puede tener diferentes impactos en la efectividad antimicrobiana del filtro. Mientras que las proteínas, lípidos o lactosa reducen la vida útil hasta un 11%, la presencia de ácidos orgánicos o alcohol la aumenta cerca de un 200%.

PALABRAS CLAVES: Óxido de silicio, antimicrobiano natural, filtración, estabilización

RESUM

La indústria alimentària urgix desenvolupar noves tecnologies per a mitigar o reduir els efectes negatius dels mètodes de conservació convencionals (com els processos tèrmics o l'ús de conservants químics). Una de les estratègies proposades recentment per reduir la càrrega microbiana de diferents productes alimentaris líquids consisteix a usar partícules de sílice funcionalitzades amb vainillina a manera de llit de filtració. No obstant això, s'ha observat que el poder antimicrobià d'aquestes partícules canvia en funció de la matriu alimentària (suc, llet, vi ...). Aquest estudi se centra a aclarir l'efecte dels components individuals de les matrius alimentàries típiques (sals, proteïnes, carbohidrats, lípids, etanol, àcids) sobre la capacitat del sistema de filtració per reduir o eliminar la càrrega microbiana inicial. Per a això, es funcionalitzaren micropartícules de sílice (50-110 µm) amb vainillina. Es van preparar diferents models d'aliments amb diferents concentracions d'albúmina sèrica bovina, sacarosa i lactosa, oli de gira-sol, àcid cítric,etanol, $MgCl_2$, $CaCl_2$ i $NaHCO_3$. Per a cada concentració, es van filtrar 100 mL de models d'aliments inoculats amb *Escherichia coli* a través d'una capa de suports funcionalitzats amb vainillina. Primer es va determinar la concentració màxima dels diferents compostos orgànics i inorgànics que es poden utilitzar fins que el filtre perd la seua activitat. Després, es va realitzar un estudi de vida útil, definit com el volum que es pot filtrar fins a perdre la capacitat antimicrobian. Els resultats van mostrar que, si bé els components inorgànics no afecten la capacitat antimicrobiana, la presència de compostos orgànics en el medi filtrat pot tenir diferents impactes en l'efectivitat antimicrobiana de filtre. Mentre que les proteïnes, lípids o lactosa redueixen la vida útil fins a un 11%, la presència d'àcids orgànics o alcohol l'augmenta prop d'un 200%.

PARAULES CLAUS: Òxid de silici, antimicrobià natural, filtració, estabilització

1. INTRODUCTION

Among the most common conventional conservation methods for drinks are thermal processes and the use of chemical preservatives. Of these, thermal processing, which aims mainly at eliminating the harmful bacteria by heat, is a treatment widely used by industries. However, apart from being cost-efficient, thermal processing exhibit several disadvantages such as the considerable losses of heat on the surfaces of the equipment and installations, reduction of heat transfer efficiency and thermal damage by overheating, due to the time required to conduct sufficient heat into the thermal center of foods (Pereira & Vicente, 2010). In addition, thermal processing causes some degree of nutritional loss, undesirable sensorial changes and loss of some functional properties (Choi & Nielsen, 2005). On the other hand, the use of chemical preservatives in food industry can have side effects on human body. In response, novel non-thermal technologies such as ultrasounds, high pressure processing, pulsed electric fields and pulsed light treatment, among others, are being proposed to inactivate microorganisms at near ambient temperatures. However, they imply several limitations that prevent their industrial application, including limited antimicrobial efficacy (Chemat *et al*., 2011), and high implementation costs (Morris *et al*., 2007).

Increasing attention has been paid to the use of natural antimicrobials such as essential oils, phenolic compounds, fatty acids, polysaccharides, proteins/peptides, enzymes, bacteriocins and bacteriophages for food biopreservation purposes thanks to their ample biocidal activity (Pisoschi *et al*., 2018; Ruiz-Rico & Barat, 2021). Among them, essential oils are really effective antimicrobials generated by plants as a protection strategy (Burt, 2004; Daglia, 2012). Their mechanism of action is related to their chemical structure, particularly to the presence of hydrophilic functional groups like hydroxyl groups and substituents on the aromatic ring, the number of double bonds and/or lipophilicity. Of these, vanillin (4-hydroxy-3-methoxybenzaldehyde), a phenolic molecule is the principal component of the bean and pod of some plants of *Vanilla* genus which is considered as GRAS. Apart from being an important flavoring agent vanillin has a proven antimicrobial activity. Vanillin has showed antimicrobial activity in apple juice and fruit purées (Cerrutti *et al*., 1997; Fitzgerald *et al*., 2004), in flavored soy beverage and yogurt (Tipparaju *et al*., 2004; Penney et al., 2004), fresh apples slices (Rupasinghe *et al*., 2006), and fresh mango slices (Ngarmsak *et al*., 2006). Presence of an own aldehyde group in the vanillin structure gives the advantage of preparing vanillin-functionalized silica supports faster compared to other essential oils like thymol, eugenol and carvacrol (Ruiz-Rico *et al*., 2017).

Despite the advantages of using essential oils, such as vanillin, as natural antimicrobials in food technology, their direct application to food can affect food properties, mainly sensory properties, and diminish efficacy due to food matrix factors, such as fat content, proteins, water activity, pH and enzymes (Lucera *et al*., 2012). However, these drawbacks can be solved by the immobilization of the aforementioned molecules on the surface of silica particles. For this, surface coating strategies have been developed to immobilize bioactive compounds on materials that can be potentially applied to diverse industrial areas (Treccani *et al*., 2013). In this context, a new antimicrobial system, based on the covalent immobilization of naturally-occurring antimicrobial molecules, such as essential oil components(EOCs), on supports has been recently developed by Ruiz-Rico *et al*., (2017). Immobilization consists of anchoring a biomolecule to a support, preserving the bioactive properties of grafted molecules, conferring the support material new properties, and preventing them from continuous circulation in the environment. It offers greater stability, longer shelf life, biomolecule metabolism prevention and biomolecule migration inhibition, all because the covalent bond between the functional groups present in the biomolecule and on the support surface does not allow the biomolecule to be released (Ruiz-Rico & Barat, 2021).

Having in mind that in beverage industry filtration is an important operation to clarify, stabilize and/or concentrate by removal of solid particles from the liquid (Fuenmayor *et al*., 2014), the incorporation of the developed particles as filtering elements to retain and inactivate microorganisms is seen as a promising strategy (Peña-Gómez *et al*., 2019a). Concretely, the proposed antimicrobial filtration technology has been recently used in different studies with variety of drinks such as wine, beer, apple juice, milk and horchata (Calabuig-Benavent *et al*., 2017; Cava-Roda *et al*., 2012; García-Ríos *et al*., 2018; Peña-Gómez *et al*., 2020; Peña-Gómez; *et al*., 2019a). The filtration of microorganism suspensions of wine through EOC-functionalized membranes showed remarkably antimicrobial activity (García-Ríos *et al*., 2018). The microbiological analysis of filtered beer showed that the supports presented remarkable removal capacity against *Escherichia coli*, mesophilic bacteria, lactic acid bacteria, mold and yeast (Peña-Gómez *et al*., 2020). Moreover, it was confirmed in the study of Peña Gómez *et al*., (2019a) that the filtration process, through the EOC-functionalized supports, allows to: (i) clarify juice to avoid turbidity and sediment in the end product; (ii) microbiologically stabilize the food matrix to increase its shelf life. In fact, treating fresh juice with this technology eliminated its native flora, and resulted in an apple juice with a much longer shelf life than that obtained by heat treatment. However, in the study of Cava-Roda *et al*., (2012) it was observed that the fat in milk reduced significantly the antimicrobial activity of vanillin. The same was observed in the study of Calabuig Benavent *et al*., (2017), in which the antimicrobial efficiency of essential oils were decreased due to the horchata matrix effect. According to studies, the application showed varying antimicrobial efficacy depending on the beverages.

This study aimed to evaluate the matrix effect on the efficacy of Vanillin-coated supports for the microbial stabilization of drinks.

2. MATERIALS AND METHOD

2.1. Chemicals

Vanillin, (3-Aminopropyl)triethoxysilane (APTES), sodium borohydride, bovine serum albumin, silica particles (50-110 µm) were purchased from Sigma-Aldrich (Madrid, Spain). Methanol, ethanol, sucrose, lactose, citric acid, NaCl, KCl, Na_2HPO_4, KH_2PO_4, HCl, $MgCl_2$, $CaCl_2$, $NaHCO_3$ and microbiological media were provided by Scharlab (Barcelona, Spain). Sunflower oil purchased in a local supermarket.

2.2. Synthesis of vanillin-functionalized silica particles

The functionalized silica particles were prepared by the covalent immobilization of vanillin on the bare silica support following the surface silanisation procedure describe by García-Ríos *et al*., (2018). The aldehyde derivative of unmodified vanillin was reacted with APTES to obtain alkoxysilane derivatives capable of being attached to silica microparticles' surface. For this purpose, firstly 40 g of the silica particles were reacted with 14 mL of the APTES in the presence of Isopropanol (200 mL) during 1 hour while stirring (400 rpm) at room temperature. Afterwards, 8 g of vanillin dissolved in 120 mL isopropanol were added. The mixture was left for another hour to stir. The obtained solid was separated by centrifugation (8700 rpm, 8 min) and mixed with 1.2 g of sodium borohydride in the presence of methanol (500 mL) overnight with the aim of reducing the formed imine bond to an amine one. Reduced vanillin-functionalized silica particles were separated by centrifugation and washed with distilled water (pH 4) until any leaching of vanillin was observed in an HPLC analysis. Finally, the solid was left to dry at 30°C for 24 hours. In **Figure 1** the synthesis procedure of the vanillin-functionalized silica support is shown.

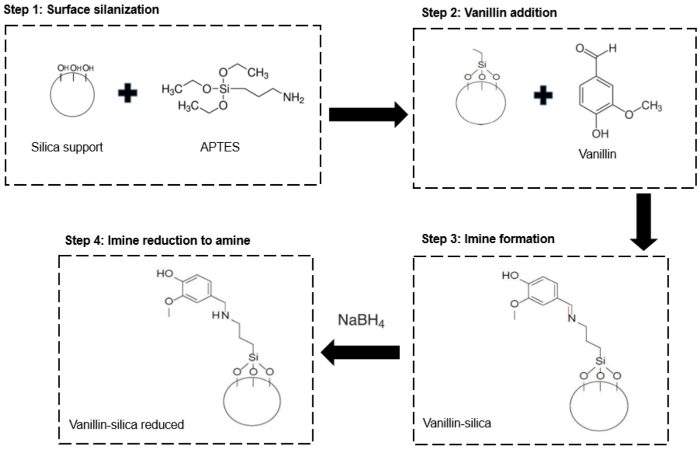

Figure 1. Representation of the synthesis procedure of the vanillin-functionalized silica particles.

2.3. Materials characterization

The starting and vanillin-functionalized silica particles were characterized using standard techniques to ensure that the solids had been correctly synthesized and functionalized. The particles' morphological analysis was performed by field emission scanning electron microscopy (FESEM) observations. FESEM images were acquired by a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. The degree of functionalization was determined by elemental analyses. Zeta potential analysis was performed in a Zetasizer Nano ZS (Malvern Instruments, UK) using previously sonicated particle suspensions in distilled water (1 mg/mL). Zeta potential values were obtained by applying the Smoluchowski model (Hunter, 1981).

2.4. Microbial assays

2.4.1. Preparation of inoculated food models

The bacterial strain used in the microbiological studies was *Escherichia coli* K12 (CECT 433, Colección Española de Cultivos Tipo, Valencia, Spain). Plate Count Agar (PCA) and Tryptone Soy Broth (TSB) were used to grow *E. coli*. The bacterial strain was reconstituted following the CECT instructions and bacterial stock was stored at 4 °C on PCA. Later, the cells from a colony were taken to a test tube with 10 mL of TSB. It was incubated at 37 °C for 24 hours to obtain an inoculum with an approximate microbial density of 10^9 CFU/mL. Phosphate-buffered saline (PBS) was used for the preparations of the decimal dilutions of the inoculum till 10^6 CFU/mL, while the last dilution 10^5 CFU/mL was prepared in the food models.

Food models were obtained by dissolving the relevant component with selected concentrations in sterile distilled water. In case of mineral salts food model, hard water conditions were stimulated taking the Spanish Standard (UNE-EN 1276-2020) as a reference, the salts of $MgCl_2$, $CaCl_2$ and $NaHCO_3$ were used.

2.4.2. Filtration assays

The filtration assays were performed in a stainless steel manifold filtration system (Microfil®, Merck Millipore, Darmstadt, Germany) connected to an Erlenmeyer flask to collect the sample. The inoculated food models were filtered through a system with three layers. These layers are as follows, from top to bottom; vanillin functionalized support (25 g), a cellulosic paper and a cellulose membrane (47 mm in diameter with a 0.45-μm pore size; Millipore, Merck), which retained the microorganisms contained in the sample. For each component and each concentration, 100 mL of inoculated food model was filtered through this system of three layers to evaluate the microbial retention capability of the vanillin-functionalized supports.

Once the results of microbial retention capability were evaluated, the assessment of the reuse capability of the vanillin-functionalized support (shelf-life of the support) was performed. For this, continuous filtration of the food model was carried out (with a selected constant concentration). During the filtration, samples were taken each 200 mL (starting from 100 mL).

During the microbial analysis of food models, the selective medium Tryptone Bile X-Glucuronide (TBX) agar was used. The plates with cellulose membranes were incubated at 37 °C for 24 hours. The values of the counts were logarithmically transformed and expressed as $\log_{10}$ CFU/mL.

Filtration assays were done in duplicates of each filtration. Controls were performed in the absence of vanillin-functionalized silica particles.

2.5. Leaching of the vanillin

The potential leaching of the immobilized vanillin was evaluated by determining the presence of vanillin in 100 mL filtered food model. The filtered food models were stored in proportions of 10 mL in Falcon tubes at 4 °C to be analyzed. The quantification of compounds in water was made by High Performance Liquid Chromatography (HPLC) following the methodology of Pérez-Esteve *et al*., (2016). Methanol and H_2O were used as mobile phase. The percentage of the leached vanillin was calculated by considering the total vanillin attached to the support. The total accumulated leached vanillin was also calculated.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial supports characterization

Figure 2 shows the morphology of non-modified and vanillin-functionalized supports performed by field emission scanning electron microscopy (FESEM). As observed, silica particles exhibit a size among 20–100 µm. No changes on the surface of the supports were detected when comparing the bare and functionalized particles, which confirmed that the immobilization process did not affect the integrity of the supports. Zeta potential of bare silica particles was calculated as -28,9 ± 0,34 mV meanwhile functionalized silica particles zeta potential value was calculated as -13,63 ± 0,84 mV. The change of the zeta potential value proves that the expected changes on the surface of the silica particles occurred after the immobilization. Finally, by using elemental analysis the amount of vanillin anchored to the particles was determined, obtaining a vanillin concentration of 4g/100 g of SiO_2.

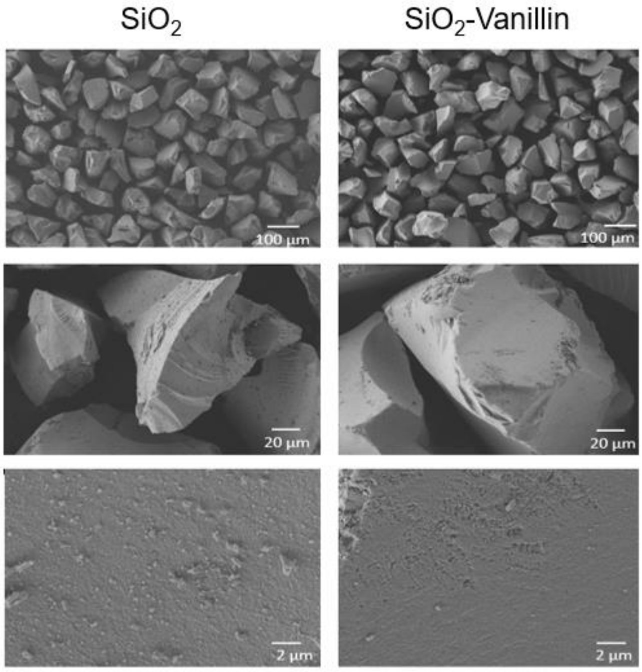

Figure 2: Characterization of particle size and particle shape by the FESEM

3.2. Matrix effect on the antimicrobial efficacy of Vanillin-coated supports

Figure 3 shows the microbial inhibition counts of *E. coli* K12 (log CFU/100 mL) after filtering 100 mL inoculated food models with different proportions of related component. The concentration 0 g/100 mL represents inoculated distilled water in each experiment. After the filtration of 100 mL of inoculated distilled water (without any organic or inorganic compound) through the bed of particles, a reduction of 2,82 ± 0,14 log CFU/100 mL was seen. After obtaining this value, considered as the control, the maximum concentration of different food matrix constituents that might be used until this antimicrobial activity disappeared was determined.

In the case of proteins, **figure 3A** shows that the antimicrobial activity could be observed until 0,01 g/100 mL of protein was used (reduction of 2,22 ± 0,001 log CFU/100 mL). With higher concentrations of protein, there were no reduction of *E. coli* load. In the same way, the antimicrobial efficacy of vanillin-functionalized support was reduced in the presence of lipids. As **figure 3B** shows, the particles lose their antimicrobial activity in the presence of a lipid content higher than 0,5 g of sunflower oil/100 mL of water. According to the Spanish General Quality Standard for UHT Milk (BOE, 1987), the minimum protein composition of milk, which is whole milk, is 2,8 g/100 mL. In this case the milk protein composition is superior to the concentration where the vanillin-coated support is still active. The same standard indicates that the lipid content of skim milk is maximum 0,3 g/100 mL meanwhile the minimum lipid content for semi milk is 1,5 g/100 mL. Therefore, only the skim milk lipid composition is between the ranges where the functionalized silica particles are still effective as an antimicrobial.

With lactose, **figure 3C**, carbohydrate of animal origin, the functionalized supports could reduce the microbial load till 3 g/100 mL of lactose (reduction of 2,24 ± 0,08 log CFU/100 mL). No reduction of the inoculated bacterium was observed with higher proportions of lactose. These values are lower than the minimum content established in milk (minimum 4,2 g lactose/100 mL milk) in the BOE (1987). Thus, taking into account the negative effect of protein, lipids and lactose on the antimicrobial activity of vanillin-coated silica particles, the low effect of this system on the microbial quality of milk observed by Mejía-Díaz (2019) is explained.

In contrast, the vanillin-functionalized supports during the filtration of solutions of sucrose, **figure 3D,** carbohydrate of plant origin, displayed remarkable antimicrobial activity till 5 g/100 mL sucrose (reduction of 2,50 ± 0,04 log CFU/100 mL). A reduction of the microbial load was observed with higher concentrations, but with less inhibition capacity.

In all the studied concentrations of citric acid before it has an antimicrobial activity by itself (2g/L), the vanillin-functionalized coat reduced the inoculated bacterium (2,69 ± 0,29 log CFU/100 mL at 2 g/L) (See **figure 3E**). The effect of the alcohol, **figure 3F**, on the efficacy of vanillin-coated supports was studied till 15% of alcohol. In this case, even the inhibition capacity was reduced with the rising of the degree of the alcohol, the functionalized supports kept having antimicrobial activity (reduction of 2,10 ± 0,03 log CFU/100 mL at 15%).

These findings, agree with the bad results found by Calabuig Benavent *et al*, (2017) when employing this system for horchata sterilization. As found in the Spanish Real Decreto 1338/1988 (BOE, 1988), pasteurized, sterilized or UHT tigernut horchata should have a minimum fat content of 2 g/100 mL. Moreover, the minimum pH would be 6.3 and the total sugars expressed as sucrose are at least 10 g/100 mL (BOE, 1988). As it can be noticed, for all the constituents, the minimum allowed concentrations are higher than the ones that negatively affect the efficiency of the antimicrobial filtering supports.

Regarding to fruit juices, the Spanish Real Decreto 1518/2007 (BOE, 2007) indicates that an apple juice can have sucrose concentration varying between 20-60 g/L and citric acid values as 6-22 g/L. According to this standard, different juices can have similar values of sucrose, while citrus fruits would have higher values of citric acid. Vanillin-functionalized supports keep having antimicrobial affect with these sucrose values. Moreover, the organic acid analysis could be performed till 2 g/L citric acid due to no microbial growth is found in solutions with higher concentrations, because of the low pH levels. Thus, these data confirm why the microbial stabilization of apple juice using this system is possible (Peña-Gómez *et al*., 2019a).

By its part, regular beers vary between 3% and 6% alcohol by volume (Missbach *et al*., 2017). Beer with low alcohol has alcohol content between 1 and 3 percent by volume and beer without alcohol has less than 1 percent by volume (Real Decreto 678/2016). Different alcoholic drinks have alcohol content between 5 to 15% such as ciders, wines, liquors, sake etc. In any case in all the concentration of alcohol analyzed the vanillin-functionalized silica particles showed antimicrobial activity. Having in mind that beer does not contain fat, neither a higher protein content, and that ethanol does not affect the antimicrobial power of the antimicrobial particles, the low matrix effect observed by Peña-Gómez *et al*., (2020) in the microbial stabilization of craft beers is explained.

3.3. Matrix effect on the antimicrobial shelf-life of Vanillin-coated supports

Figure 4 shows the microbial inhibition counts of *E. coli* K12 (log CFU/100 mL) after filtering different increasing quantities of each of the studied food models through the vanillin-functionalized support to assess the antimicrobial shelf-life of the support. For these studies, for each of the food constituents it was used the higher concentration that do not inhibit the antimicrobial power of the particles bed (see section 3.1).

As shown in **figure 4A,** the lowest reuse capability of the vanillin-functionalized silica particles was found using proteins at a concentration of 0,01 g/100 mL. In this case, the solid lost its antimicrobial powder after filtering the first 100 mL of protein solution (inhibition count of 1,98 ± 0,027 log CFU/100 mL).

With lipid (1,5 g/100 mL), **figure 4B**, and lactose (2,5 g/100 mL), **figure 4C,** the filter also displayed a low reuse capability by having antimicrobial activity only till 300 mL of solution with both cases (reduction of microbial load is 1,98 ± 0,02 log CFU/100 mL for lipid solution, 2,03 ± 0,08 log CFU/100 mL for lactose solution at 300 mL).

During the analyses the limits of the reuse capability of the functionalized silica particles were also determined for sucrose (5 g/100 mL), **figure 4D**, the mixture of alcohol (12%) and sucrose (5 g/100 mL), **figure 4G**, and the mixture of mineral salts ($MgCl_2$, $CaCl_2$, $NaHCO_3$), **figure 4E**. For sucrose a significant reduction of the microbial load was observed till 700 mL (2,22 ± 0,11 log CFU/100 mL at 700 mL). Less reduction was seen with 900 mL (1,17 ± 1,17 log CFU/100 mL at 900 mL) and finally no inhibition was displayed for further quantities. No antimicrobial activity was seen for 1500 mL and further quantities for the mixture of alcohol and sucrose. It presented a clear reuse capability till 1100 mL (2,23 ± 0,12 log CFU/100 mL at 100 mL). With the mineral salts inhibition of *E. coli* load occurred till 900 mL (1,98 ± 0,02 log CFU/100 mL). For further quantities no inhibition was shown.

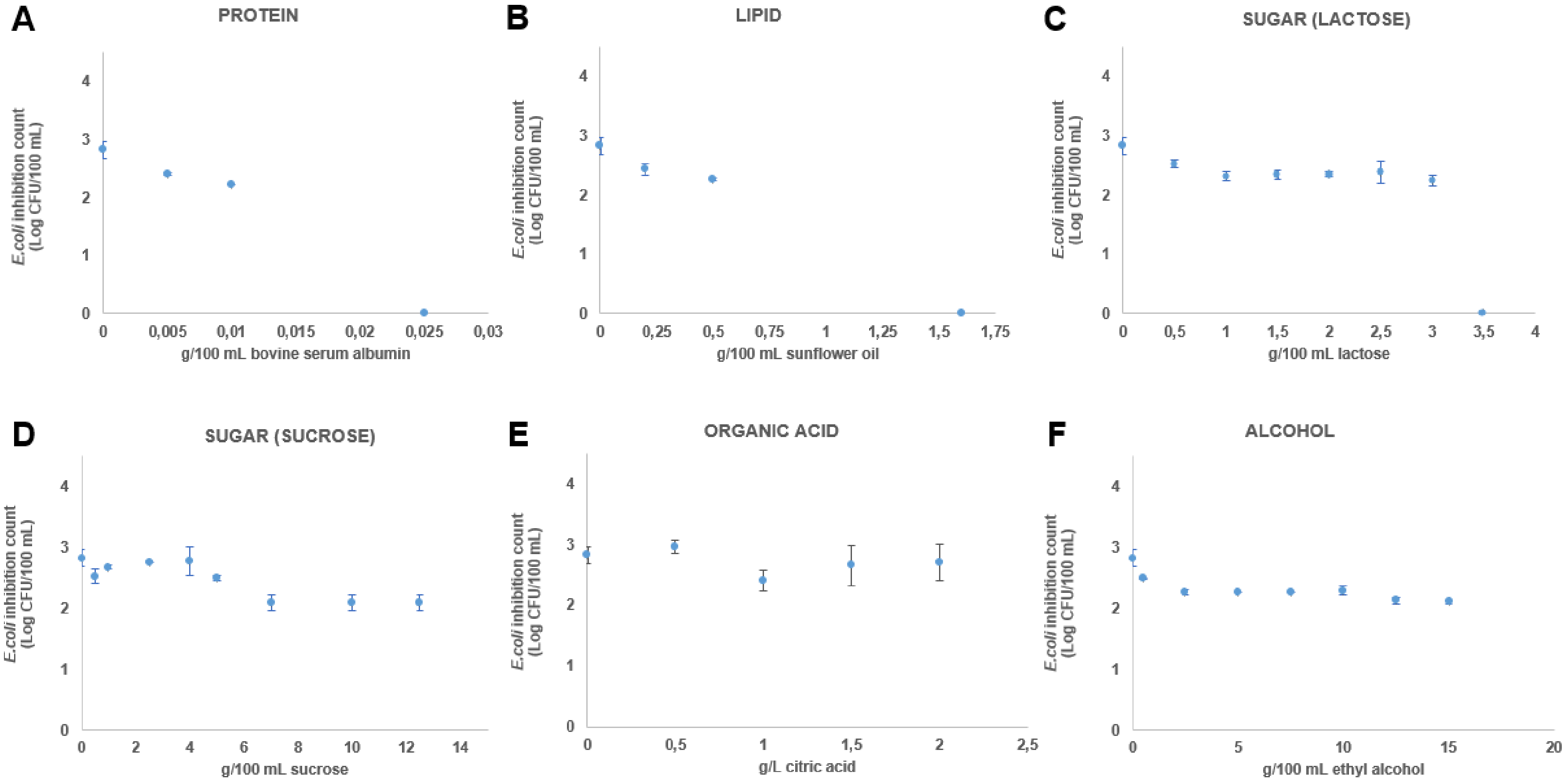

Figure 3. Microbial inhibition counts (log CFU/mL) of *E. coli* K12 after filtering inoculated food models with different proportions of (A) protein (B) lipid (C) lactose (D) sucrose (E) citric acid (F) alcohol.

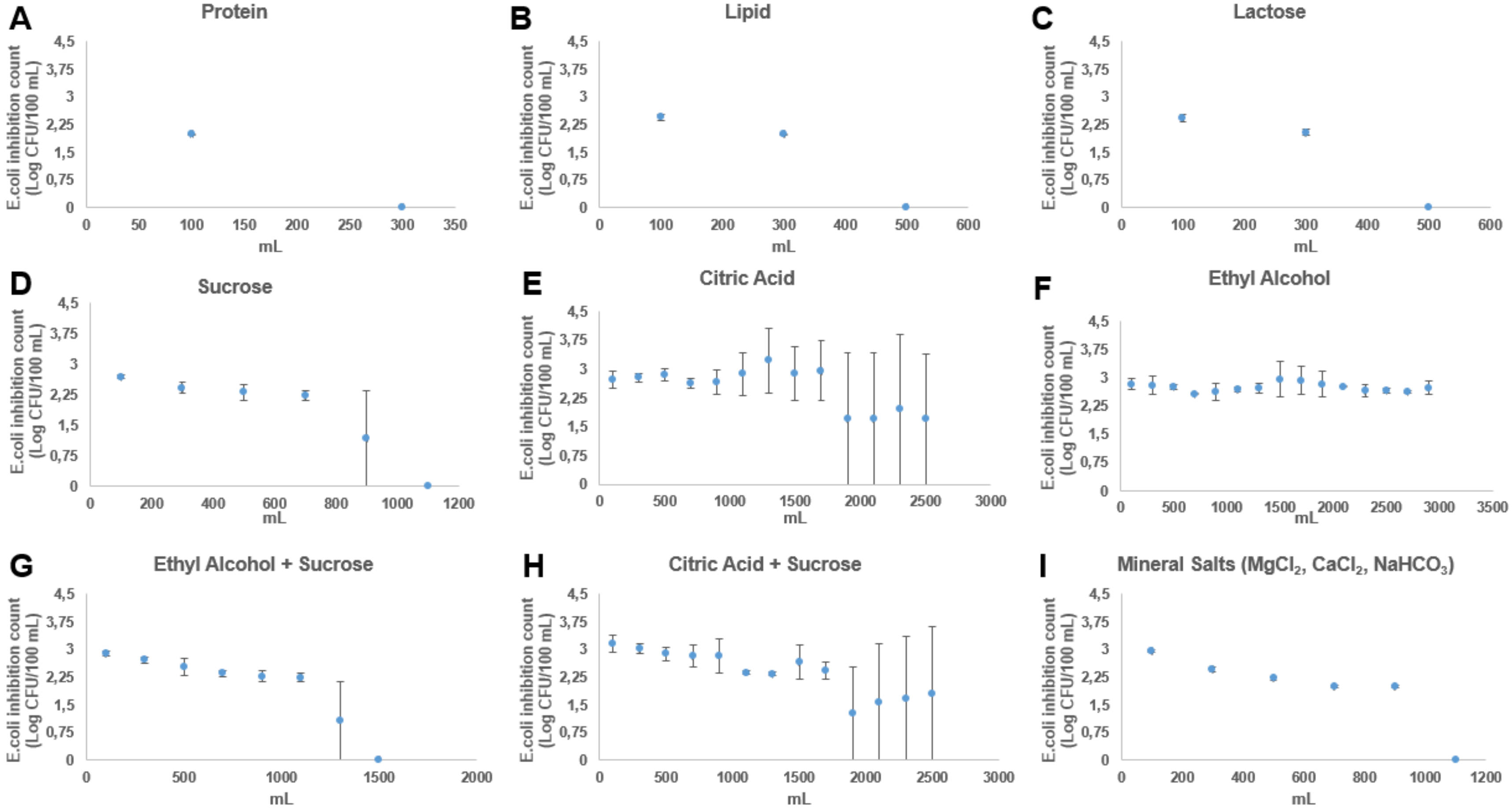

Figure 4. The microbial inhibition counts of *E. coli* K12 (log CFU/mL) after filtering the food models to assess the reuse capacity of the vanillin-functionalized support. (A) 0,01 g/100 mL bovine serum albumin (B) 0,5 g/100 mL sunflower oil (C) 2,5 g/100 mL lactose (D) 5 g/100 mL sucrose (E) 2 g/L citric acid (F) 12% ethyl alcohol (G) 12% ethyl alcohol and 5 g/100 mL sucrose (H) 2 g/L citric acid and 5 g/100 mL sucrose (I) Hard water conditions stimulated as described in Spanish Standard UNE-EN 1276-202

These results are again in agreement with previous studies. Filtration through the essential oil compounds-functionalized supports played a significant role in the elimination of the *E. coli* inoculated in the apple juice samples (Peña-Gómez, 2019a). Therefore, sucrose analyses displayed the results expected, it showed promising results as well as organic acid solutions. The results show that the mixture of both compounds improved the reuse capability of vanillin-functionalized support. The decrease of the pH level due to addition of citric acid might have created a synergistic effect and improve the antimicrobial effect of the silica particles. Similar explanation can be given for the mixture of alcohol and sucrose. As seen in the previous studies the antimicrobial support showed effectiveness with the concentrations representing the alcohol degrees of beer.

The use of vanillin-functionalized silica particles showed promising results in applications with wine (García-Ríos *et al*., 2018).The antimicrobial device displayed lower antimicrobial activity with concentrations of alcohol representing wine but stayed effective during the reuse capability study even after 3000 mL of food model filtered.

To show clearly the comparison between the effect of the different compositions on the shelf-life (reuse capability) of the vanillin-functionalized particles, **figure 5** has been made. Taking filtration of pure water as a reference for the 100% capacity, components like protein, lipid, lactose or sucrose decreased the shelf-life of the functionalized coat. Meanwhile the presence of alcohol or citric acid improved the reuse capability of the functionalized silica particles. It can be observed that the addition of sucrose to the acid solution didn't have significant effect in comparison with the acid solution. But the silica particles showed less reuse capability with the addition of sucrose to the alcohol solution in comparison with the alcohol solutions only. Between all the components studied, the functionalized particles showed the best performance with alcohol, citric acid and the mixture of citric acid-sucrose but the reuse capability limit of the functionalized support has not been defined yet for these compositions as the particles kept being active after the quantity of solutions filtered.

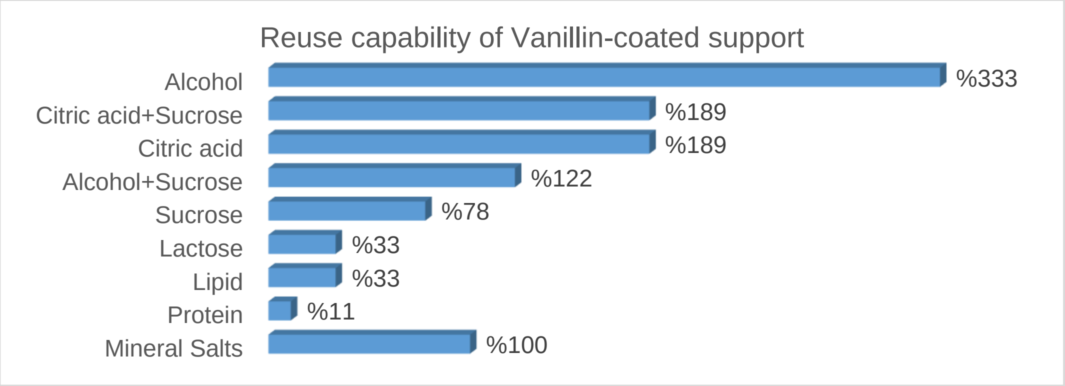

Figure 4. Reuse capability of Vanillin coated-support for inhibition counts of *E. coli* K12 (log CFU/mL)

3.3 Leaching of vanillin

It has been found that the aldehyde group in vanillin is the main responsible for its antimicrobial effect despite the influence of the type and the position of some of the lateral groups in the benzene ring (Fitzgerald *et al.*, 2005). The antimicrobial mechanism is mainly based on its ability to destroy the plasmatic membrane of the microbial cells through interaction with the lipids or proteins, or with both structures, with a subsequent loss of the ionic gradient and inhibition of the respiratory activity due to its hydrophobic nature (Cava-Roda *et al*., 2012).

Having this in mind, one possible explanation of the loss of antimicrobial power might be the break of the imine bond between the silica particle and the vanillin and the leaching of the active compound. In this sense, to define the stability of the immobilization procedure and to determine the wash-out effects depending on the composition of the liquid and the quantity filtered, the potential leaching of the vanillin was quantified by using HPLC. **Table 1** shows the percentage of lixiviated vanillin in food models and the percentage of the total accumulation of leached vanillin after filtering through the vanillin-functionalized supports.

Observing the total accumulated compound percentage, the biggest loss of vanillin compound happened with the filtration of citric acid. The compound kept leaching till 900 mL and in total 0,150 ± 0,008 percentage of anchored vanillin has been wash-out. Following the citric acid results, sucrose solution and the mixture of citric acid with sucrose showed similarities, in both cases the leached vanillin could be detected till 700 mL of filtering.

Food models containing only sucrose has caused a total leaching of 0,10 ± 0,05 percent, while vanillin wash-out accumulation was calculated as 0,08 ±0,08 percent of the attached vanillin with the mixture of citric acid and sucrose.

Lactose food models also cause leaching till 700 mL but the total wash-out has only reached 0,059 ± 0,08 percent of the anchored vanillin to the silica particles.

Food models containing only alcohol and food models containing both alcohol and sucrose displayed similar results. The leaching continued till first 500 mL and reached to 0,04 ± 0,06 percent of loss of anchored vanillin.

Mineral salts solution which stimulated the conditions of hard water caused very low quantity of leaching in comparison with the rest of the food models. In this case, the leaching could be detected only after the filtering of first 100 mL and it was calculated as 0,011 ± 0,016 percent of the vanillin immobilized.

During the filtration of protein and lipid food models, a zero wash-out of vanillin was detected (values below the detection limit).

Table 1. Vanillin leaching (mg), the relative percentage and the percentage of the total accumulation of the leached vanillin after filtering the food models through the bed of the vanillin-functionalized supports.

Component	**Filtered quantity (mL)**	**Leached compound (mg released Vanillin/ mg Vanillin anchored)**	**Total accumulated compound (mg released Vanillin/ mg Vanillin anchored)**
Citric acid	100	0,044 ± 0,006	0,15 ± 0,008
	300	0,050 ± 0,001	
	500	0,03 ± 0,004	
	700	0,013 ± 0,002	
	900	0,013 ± 0,00	
	1100	ND	
Citric acid and Sucrose	100	0,027 ± 0,009	0,08 ± 0,08
	300	0,033 ± 0,030	
	500	0,019 ± 0,027	
	700	0,007 ± 0,010	
	900	ND	
Sucrose	100	0,039 ± 0,018	0,10 ± 0,05
	300	0,032 ± 0,016	
	500	0,022 ± 0,011	
	700	0,0096 ± 0,014	
	900	ND	
Lactose	100	0,026 ± 0,037	0,059 ± 0,08
	300	0,015 ± 0,022	
	500	0,012 ± 0,018	
	700	0,006 ± 0,009	
	900	ND	
Ethyl alcohol	100	0,023 ± 0,034	0,04 ± 0,06
	300	0,013 ± 0,019	
	500	0,006 ± 0,009	
	700	ND	
Ethyl alcohol and Sucrose	100	0,019 ± 0,027	0,04 ± 0,06
	300	0,013 ± 0,020	
	500	0,007 ± 0,011	
	700	ND	
Mineral Salts	100	0,011 ± 0,016	0,011 ± 0,016
	300	ND	
Lipid	100	ND	0
Protein	100	ND	0

ND (not detected)

The low percentage of vanillin leached during the essays confirms that the antimicrobial power inhibition is not due to a leaking of the vanillin in the media. However, vanillin might also interact with hydrophobic constituents (lipids and proteins) of complex food systems, such as milk, with diminished antimicrobial effectiveness (Cava-Roda *et al*., 2012; Shah *et al*., 2012). This can explain the reason why the vanillin-functionalized particles are not effective with the food models prepared with lipid and protein even with considerably low concentrations. The formation of a protective film around the bacterial cells with lipid might also prevent the antimicrobial action (Mejlholm & Dalgaard, 2002).

In previous studies, apple juice (containing organic acids and carbohydrates of plant origin) filtered through the vanillin-functionalized support showed a 0.6% of the initial compound's content grafted to particles' surface (Peña-Gómez *et al*., 2019a). No wash-out effects after filtration of water resulted in the stable essential oil components bonding on the supports' surface for vanillin (Peña-Gómez *et al*., 2019b).

Finally, it must be said that the presence of the vanillin is considered GRAS at low concentrations (Hyldgaard *et al*., 2012).

4. CONCLUSIONS

This study has revealed the importance of studying the composition of the food matrix to determine the antimicrobial efficacy of a silicon oxide particle functionalized with essential oil components. In this case, for vanillin as antimicrobial molecule, it was observed that the protein, lactose, or lipid presence in the liquid decreased remarkably the efficacy and the shelf-life of the antimicrobial supports. Meanwhile, the presence of citric acid or alcohol in the liquid, did not only reduce the antimicrobial poser of the bed of particles, but also increased the reuse capability when using binary mixtures with sugars (sucrose-alcohol or sucrose-citric acid). These results, beyond justifying previous studies, are a starting point to predict the efficiency of this antimicrobial system in other food matrices. However, to be able to understand the working mechanism of vanillin-coated supports and develop new application fields in the industry it is necessary to study the retention capability against other spoilage and pathogenic microorganisms as well as the influence of the nature of the type of antimicrobial used (terpene, organic acid, fatty acid…).

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