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Ruiz Rico, M.; Renwick, S.; Allen-Vercoe, E.; Barat Baviera, JM. (2022). In vitro susceptibility of human gut microbes to potential food preservatives based on immobilized phenolic compounds. Food Chemistry. 378:1-10. https://doi.org/10.1016/j.foodchem.2022.132136

The final publication is available at

https://doi.org/10.1016/j.foodchem.2022.132136

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Additional Information

***In vitro* susceptibility of human gut microbes to potential food preservatives based on immobilized phenolic compounds**

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Abstract

The development of novel food preservatives based on natural antimicrobials such as phenolic compounds is increasing, but their safety should be established before use, including evaluating their impact on the gut microbiota. This work explored the influence of antimicrobial phenolics presented in different forms on selected human gut microbiota members through *in vitro* susceptibility tests. The bacteria tested exhibited a wide range of susceptibilities to phenolics depending on the molecule structure and mode of administration. *Agathobacter rectalis* and *Clostridium spiroforme*, members of the phylum Firmicutes, were the most sensitive strains. Susceptibility was strain- and species-specific, suggesting that it may not be possible to easily extrapolate results across the human microbiome in general. Species of other phyla including Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia were more resistant than Firmicutes, with growth of some strains even enhanced. Our results provide insights into the biocompatibility of free and immobilized phenolics as potential food additives.

Keywords: gut microbiota, natural antimicrobial, covalent immobilization, food additives, *Agathobacter*, *Clostridium*

1. Introduction

Food additives are substances incorporated into almost all processed food. Several lines of evidence suggest that some food additives may contribute to gut microbiota alterations, although further research is needed (Cao et al., 2020). The impact of food additives such as artificial sweeteners, emulsifiers, colorants and preservatives on the gut microbiota has been evaluated in recent years, indicating that these substances could induce imbalances in ecosystem composition. Changes in the gut microbiota can affect human health and have been associated with chronic bowel disorders, systemic and targeted inflammation, metabolic syndrome, and neurological diseases (Rinninella, Cintoni, Raoul, Gasbarrini, & Mele, 2020).

The influence of artificial preservatives on the gut microbiota has recently been evaluated. Studies found a significant impact of exposure to sodium benzoate, sodium nitrite, or potassium sorbate on single gut microbiota species as well as mice colonized with a human-derived microbiome, the latter resulting in an overgrowth of intestinal bacteria with proinflammatory properties and a decrease of commensal beneficial bacteria (Hrncirova, Hudcovic, et al., 2019; Hrncirova, Machova, Trckova, Krejsek, & Hrncir, 2019).

Given the current safety issues related to the use of artificial preservatives (Keeton, 2011), different naturally-occurring antimicrobial compounds are being proposed as alternative additives (Pisoschi et al., 2018). Among natural antimicrobials, several phenolic compounds from essential oils or byproduct from aqueous plant extracts have been recognized to have bacteriostatic or bactericidal properties and could potentially be used as preservatives (Faustino et al., 2019; Gutiérrez-del-Río, Fernández, & Lombó, 2018). Studies of phenolic compounds from dietary polyphenols have shown that these compounds may modulate the composition of the microbiota through selective prebiotic effects leading to stimulation of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, as well as antimicrobial activities against gut pathogenic bacteria (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; Ma & Chen,

2020; Ozdal et al., 2016). However, the impact of the phenolic compounds on the gut microbiota depends on their chemical characteristics, including antimicrobial activity, bioavailability and utilization as metabolic substrate by microbiota (Wlodarska, Willing, Bravo, & Finlay, 2015).

The direct application of these natural antimicrobial compounds as additives in foodstuff is limited mainly by their strong sensory properties and, therefore, current research is focused on the development of methodologies such as encapsulation or immobilization to improve their functionality (El-Saber Batiha et al., 2021). In particular, our research group focuses on the design of new antimicrobial systems based on the covalent immobilization of natural antimicrobial compounds on inert supports (Ruiz-Rico et al., 2017). The supports developed have shown good immobilization performance and were able to preserve or even improve antimicrobial activity against different microorganisms of interest to the food industry. As well, immobilization had the benefit of preventing the spontaneous release of antimicrobial substances into the medium, thus avoiding their absorption in the digestive tract (Ribes, Ruiz-Rico, Pérez-Esteve, Fuentes, & Barat, 2019).

Once the efficacy of food preservatives has been established, their biocompatibility should be verified as a prior step to their actual application in the food industry; this should be done through toxicity studies both in cell culture and mammalian models, and, as well, any effects of these food additives on the intestinal microbiota should be determined both *in vitro* and *in vivo*. However, evaluation of the effects of food additives on the gut microbiota is still only rarely done (Hrncirova, Hudcovic, et al., 2019). Therefore, the aim of this work was to evaluate the impact of natural phenolic compounds (eugenol, ferulic acid and vanillin) presented in two administration forms (either free or immobilized on different carriers) on human gut-derived bacterial isolates. To do this, *in vitro* susceptibility tests were performed with representative commensal species from defined gut microbiota communities.

2. Materials and methods

2.1. Reagents

10-μm amorphous silica microparticles (AS10), microcrystalline cellulose particles (C), *N*-cetyltrimethylammonium bromide (CTABr), tetraethylorthosilicate (TEOS), triethanolamine ($TEAH_3$), (3-aminopropyl)triethoxysilane (APTES), formic acid, trimethyl orthoformate, eugenol (99% w/w) (EU), trans-ferulic acid (99% w/w) (FE), dimethyl sulfoxide (DMSO) and NaOH were provided by Sigma-Aldrich (Madrid, Spain). 5-μm amorphous silica microparticles (AS5) was supplied by Silysiamont (Milano, Italy). Vanillin (99% w/w) (VA) was provided by Ernesto Ventós S.A. (Barcelona, Spain). 2-propanol was supplied by Labkem (Barcelona, Spain) and acetonitrile was acquired from Scharlab (Barcelona, Spain). *N*-hydroxysuccinimide (NHS) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) were supplied by Acros Organics (New Jersey, USA).

Modified Peptone Yeast extract Glucose Broth (PYGB) was prepared according to the protocol described in Table S1, supplementary information. Fastidious anaerobe agar was supplied by Neogen Corporation (Lansing, USA) and defibrinated sheep's blood was provided by Hemostat Laboratories (Dixon, USA).

2.2. *Synthesis of silica and cellulose supports functionalized with phenolic compounds*

Synthesized mesoporous silica microparticles MCM-41 (M), commercial silica gel microparticles AS5 and AS10, and cellulose microparticles were used as inert supports for the immobilization of the natural phenolics with antimicrobial properties EU, VA and FE, resulting in the phenolic-functionalized materials.

The synthesis of MCM-41 particles was carried out following the 'atrane route', with a molar ratio of the reagents: 7 $TEAH_3$: 2 TEOS: 0.52 CTABr: 0.5 NaOH: 180 H_2O. A solution of NaOH and $TEAH_3$ was heated to 120ºC and stirred at 300 rpm. The mixture was cooled to 70ºC before adding TEOS, and then the temperature was increased to

118ºC. CTABr was added to the solution, the temperature was reduced again to 70ºC and then 180 mL of deionized water were added, increasing the stirring speed to 600 rpm. A white precipitate was formed and the solution was kept in agitation for 1 h at room temperature. The suspension was then placed in a Teflon container at 100ºC for 24 h. Subsequently, the mixture was vacuum filtered, and the solid was washed with distilled water to neutral pH. The material obtained was dried at 72ºC for 24 h and calcined at 550ºC in an oxidant atmosphere for 5 h to remove the organic template.

Surface salinization was the approach used for the covalent immobilization of the different phenolic compounds but the grafting procedure was specific for each compound (see Scheme S1, supplementary information, for details). For the immobilization of eugenol (Scheme S1A), the synthesis of an aldehyde derivative by the Reimer-Tiemann reaction was firstly performed. As second step, the alkoxysilane derivative was prepared by reaction of APTES with the aldehyde of EU in a 1:1 molar ratio using 2-propanol as solvent. The mixture was refluxed for 1 h at 60ºC. Next, the derivative was covalently grafted to the hydroxyl groups present on the surface of the supports. In a typical synthesis, 40 g of supports were suspended in 200 mL of 2-propanol and reacted with the alkoxysilane derivative under orbital stirring for 3 h, followed by centrifugation and washing with water and 2-propanol (Ruiz-Rico et al., 2017).

For the immobilization of vanillin (Scheme S1B), APTES was firstly attached to the surface of the particles by reaction of 40 g of supports and APTES (2 mmol/g solid) in 200 mL of 2-propanol under orbital stirring for 1 h. Then VA, previously solved in 2-propanol, was added to the suspension (molar ratio APTES:VA, 1:1) to perform the covalent immobilization of the phenolic compound. The mixture was maintained under orbital stirring for 1 h. The final reaction in the synthesis was a reductive amination between aldehyde of VA and amine of APTES. For that, a Leuckart–Wallach reaction was carried out by using formic acid (1.5 mmol/g) as the reducing agent and adding trimethyl orthoformate (1 mmol/g) to facilitate driving the reaction to completion (Frederick & Kjell, 2015). The mixture was refluxed for 1 h at 60ºC. After cooling the

reaction, the particles were recovered by centrifugation and washed with water and 2-propanol.

For ferulic acid grafting (Scheme S1C), APTES was firstly grafted to the surface of the particles and then FE was immobilized on the supports via an amidation reaction. In a typical synthesis, 40 g of supports were suspended in 200 mL of acetonitrile and reacted with APTES (2 mmol/g) under orbital stirring at room temperature for 1 h. FE (1 mmol/g) was reacted with NHS (0.3 mmol/g) and EDC (0.3 mmol/g) in acetonitrile at room temperature for 15 min to activate the carboxylic acid before reaction with the amine moieties of APTES. The mixture was added to the particle suspension and stirred at room temperature for 24 h, followed by centrifugation and washing with water and 2-propanol. In all cases, the particles were dried at room temperature in vacuum for 12 h.

2.3. *Characterization of phenolic-functionalized supports*

The bare and phenolic-functionalized particles were characterized using three techniques: dynamic light scattering (DLS), zeta potential and elemental analysis. Particle size distribution was analyzed by DLS using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) applying the Mie theory (refractive index of 1.45, absorption index of 0.1 for MCM-41 particles and 0.001 for the other supports). Surface charge was determined by zeta potential analysis using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The electrophoretic mobility measurements were converted into zeta potential values by the Smoluchowsky mathematical model. For DLS and zeta potential, particles were suspended in deionized water and sonicated for 2 min in order to prevent the aggregation of the particles. Degree of functionalization was established by elemental analysis for C, H and N in a CHNOS Vario EL III model (Elemental Analyses System GMHB, Germany). All the analyses were conducted in triplicate.

2.4. Bacterial isolates from a defined microbial ecosystem

10 bacterial species (Table S2, supplementary information) representative of the main phyla of a defined gut microbiota community were used for exposure studies. The defined community consisted of 69 species representative of microbes native to the common human gut, and derived from a stool sample from a healthy donor (Donor A) (Petrof et al., 2013). Once the strains that were most susceptible to the test compounds were established, further assays were performed with taxonomically closely related isolates from Donor A or from other donors (B and C) (see Table S3, supplementary information, for details).

Strains were grown from frozen stocks on fastidious anaerobe agar supplemented with 5% defibrinated sheep's blood under anaerobic conditions (90% N_2, 5% CO_2, 5% H_2) in an Anaerobe Chamber (Anaerobe Systems, USA). Before their use, the species identity of each isolate was verified using 16S rRNA gene Sanger sequencing of the V3-V4 variable region of the 16S rRNA gene at the University of Guelph Advanced Analysis Centre.

2.5. Growth curves of bacterial isolates in response to free and immobilized phenolic compounds

The susceptibility of the selected bacterial isolates to the different forms of presentation of the phenolic compounds was tested using a range of concentrations (0.25, 0.5, 1 and 2 mg/mL) which were chosen based on previous studies with free and immobilized phenolic compounds (Ruiz-Rico et al., 2017; Verdú et al., 2020).

For the preparation of phenolic compounds stock solutions, different volumes of concentrated solutions of EU, FE and VA prepared in DMSO were added to PYGB. The solutions were prepared immediately prior to use. For the inoculum preparation, a single colony of the strain was suspended in 5 mL of PYGB and incubated at 37ºC under anaerobic conditions for 24 h.

The bacterial growth was monitored using a 96-well plate. Microplate wells were filled with the required volume of broth and antimicrobial stock solution to obtain 190 μL volumes in each well with the target concentrations of the free phenolic compounds. Then, 10 μL of the inoculum was added to each of the wells and the microplate was incubated anaerobically at 37°C in a plate reader (Epoch 2 Microplate Spectrophotometer, BioTek, USA) with continuous agitation. Growth measurements (OD_{600}) were automatically recorded every 30 min over a 48 h-period. The area under the curve (AUC) from average OD_{600} data (biological triplicate) from x = 0–48 using a baseline of y = 0 was calculated, through the use of the R package growthcurver (Sprouffske & Wagner, 2016). Relative AUC values were calculated in accordance to control condition without treatment.

To evaluate the impact of the immobilized phenolic compounds, the assay was modified to include a previous exposure of the strains to the functionalized particles in conical tubes. For these tests, the strains were incubated anaerobically with orbital stirring in gas jars placed within a shaking incubator (Minitron, Infors HT, Switzerland) in the presence of the particles at 37ºC for 24 h in conical tubes with 2.5 mL of PYGB and the amount of particles required to study equivalent antimicrobial concentrations (based on the content of phenolics grafted on the surface of the supports determined by elemental analysis). After exposure, samples were centrifuged at 1500 rpm for 2 min to sediment the particles and then the growth of the compound-exposed strain was further monitored using a similar 96-well plate assay as above, with 10 μL of the exposed supernatant (containing bacterial cells) placed into fresh PYGB before incubation for 48 h at 37°C under anaerobic conditions (García-Ríos, Ruiz-Rico, Guillamón, Pérez-Esteve, & Barat, 2018).

For each experimental series, control inoculated wells without phenolic compounds were included to monitor the growth of the strain in absence of treatment, and non-inoculated wells (with or without the phenolic compounds) were also included in the microplate to determine and allow subtraction of any noise signal. In addition, inoculated

samples with added DMSO (to 0.8% v/v) and non-modified supports were included to assess any potential growth effects their presence may have had on each strain. All experiments were carried out in triplicate.

2.6. Statistical analysis

The data acquired from the characterization of phenolic-functionalized supports was analyzed by a one-way analysis of variance (ANOVA) test. Significance of impact of phenolic compounds on microbial growth was determined by a one-way ANOVA to compare results with control and a multifactor ANOVA to evaluate the influence of the form of presentation of the phenolic compound and the strain tested, as well as their interaction. The least significance procedure was followed to test for differences between averages at the 5% significance level. The results were statistically processed by the Statgraphics 18 (Statpoint Technologies, Inc., Warrenton, USA).

3. Results and discussion

3.1. Characterization of phenolic-functionalized supports

Synthesizing the antimicrobial particles made it possible to obtain 12 different supports by immobilizing biomolecules (EU, VA and FE) on the surface of siliceous particles (M, AS5 and AS10) and cellulosic particles (C). The phenolic compounds were covalently grafted on the surface of the supports by surface silanization obtaining the phenolic-functionalized supports.

Figure S1, supplementary information shows the particle size distribution of the bare and phenolic-functionalized supports. These supports had particle sizes on the micrometric scale. The smallest particles were the M material with a $d_{0.5}$ value of 0.7 μm for the non-functionalized particles. Unmodified C particles had a $d_{0.5}$ value of 4.6 μm, similar to AS5 particles with a $d_{0.5}$ of 3.6 μm. Finally, the material with the largest particle size was AS10 with a $d_{0.5}$ value of 8 μm for non-functionalized particles. After the functionalization process, most of the supports maintained a particle size distribution

similar to that of the unmodified materials, demonstrating that the immobilization process did not significantly affect the morphology of the particles.

Regarding the surface charge of the particles, Figure S2, supplementary information, shows the zeta potential values of the different materials developed compared to the bare supports. Non-functionalized particles displayed negative zeta potential values between -14 and -38 mV due to the presence of deprotonated hydroxyl groups on the surface of cellulosic and siliceous materials in aqueous solution. After functionalization with phenolic compounds using an organosilane with an amine functional group (positively charged), a change is observed in the zeta potential values, becoming positive zeta potential values in most cases, as a result of the immobilization of the phenolic-organosilane derivative on the surface of materials.

The content of organic matter present on the surface of the functionalized particles was determined by elemental analysis and is shown in Table S4, supplementary information. Both the results of zeta potential and the results of elemental analysis confirm the immobilization of the phenolic compounds on the surface of the different support materials.

3.2. Free and immobilized phenolic compounds influence the growth of bacterial gut isolates

Phenolic compounds including volatile phenols such as EU, phenolic aldehydes such as VA, and hydroxycinnamic acids such as FE were chosen in this study because of their reported antimicrobial properties against food spoilage and foodborne microorganisms that promote their potential use as food preservatives (Marchese et al., 2017; Shi et al., 2016; Yang et al., 2021). In addition, the exposure of the tested gut isolates to these antimicrobials was performed using the different presentations of the phenolic compounds in order to evaluate the influence of the carrier on the impact on the gut microbiota.

For the first stage of evaluating the impact of phenolic compounds on human gut microbes, 10 species (Table S2, supplementary information) were chosen from different phyla of a defined gut ecosystem. The main phyla that reside in the human gastrointestinal tract are Firmicutes (FIR) and Bacteroidetes (BAC) (up to 90%), and at lower percentages, Proteobacteria (PRO), Actinobacteria (ACT) and Verrucomicrobia (VER). The abundances of the predominant phyla in the gut may vary individually, but the proportions in most people are similar (Cao et al., 2020). The Firmicutes phylum contains several prominent genera, including *Lactobacillus*, *Clostridium*, *Ruminococcus*, *Roseburia*, and *Eubacterium*; the Bacteroidetes phylum includes the genera *Parabacteroides, Prevotella*, and *Bacteroides;* the Actinobacteria phylum contains *Collinsella* and *Bifidobacterium*; the Proteobacteria phylum includes *Escherichia* and *Desulfovibrio* spp.; and the Verrucomicrobia phylum comprises the genera *Akkermansia* (Catalkaya et al., 2020).

The impact of the different forms of administration of the phenolic compounds on the growth of representative strains of the gut microbiota community is shown in Figure 1. This figure represents the relative AUCs for each the 10 microorganisms, compared to the control condition, for the highest concentration used in this study (2 mg/mL) of the free or immobilized phenolic compounds. The phenolic compounds produced a different impact depending on the molecule structure, bacterial strains and form of administration. Either bacteriostatic or bactericidal effects as well as growth enhancement effects were seen in tested bacterial strains after exposure in comparison to control ($p<0.001$). Gram-negative bacteria (*B. caccae, P. distasonis, E. fergusonii* and *A. muciniphila*) were generally more resistant to phenolic compounds than Gram-positive microorganisms, probably due to the differences observed in their cell wall composition (Hervert-Hernández & Goñi, 2011).

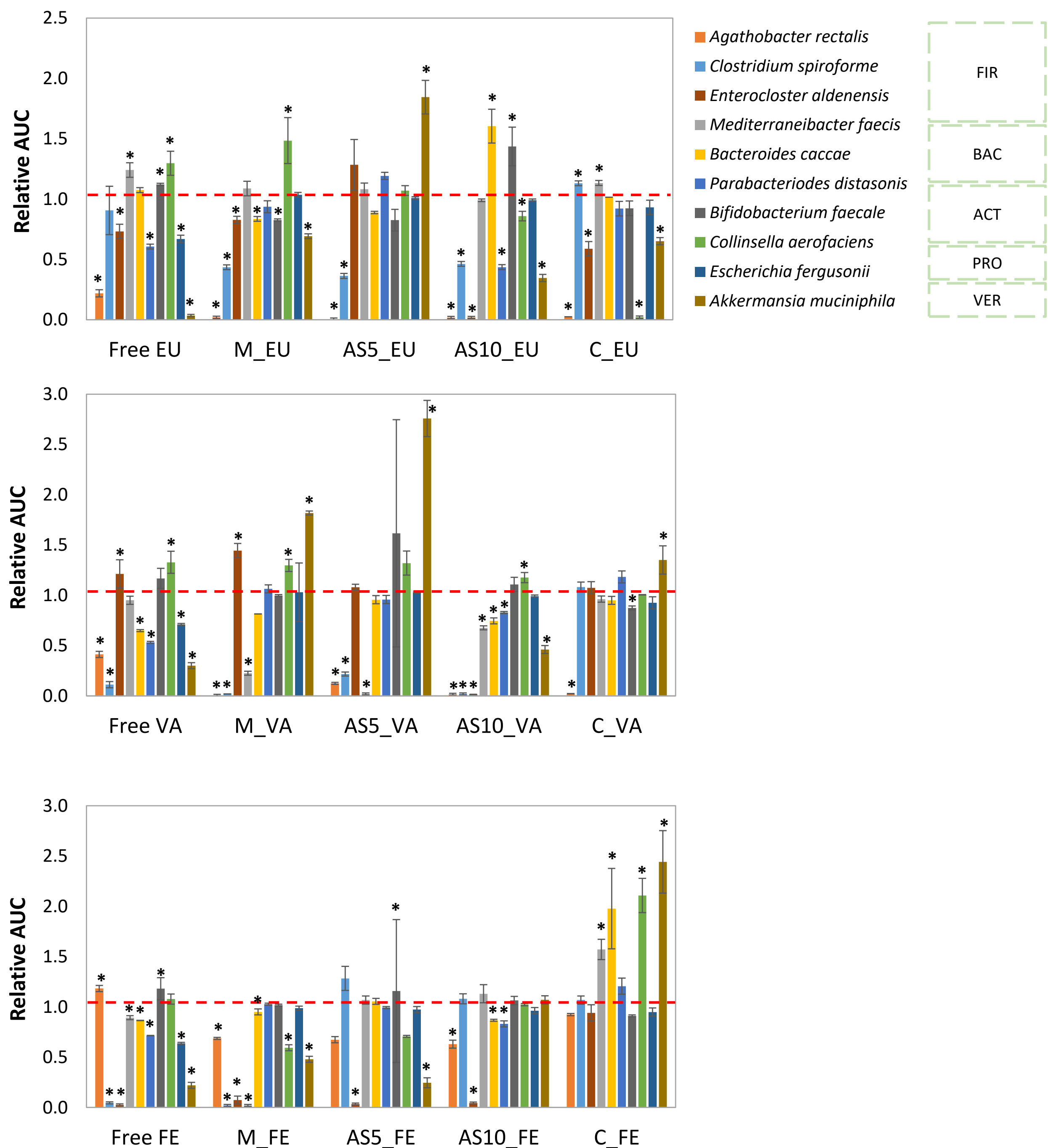

Figure 1. Relative AUC of the bacterial gut isolates after incubation with eugenol (EU), vanillin (VA) and ferulic acid (FE) administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 μm-amorphous silica particles (AS5), 10 μm-amorphous silica particles (AS10) and cellulose particles (C) (means and standard deviations, n=3). The values are relative compared with the control condition without treatment. Antimicrobial concentration of 2 mg/mL. (*) *p<0.001* indicates significant differences compared to the control.

A considerable variation in susceptibility to phenolic compounds among tested gut isolates was found, with some of the tested Firmicutes species (*A. rectalis* strain 16-6-I 1 FAA*, C. spiroforme* strain 16-6-I 21 FAA*, E. aldenensis* strain 16-6-S 15 LS and *M. faecis* strain 16-6-I 30 FAA) showing the highest sensitivity. *A. rectalis* (formerly *Eubacterium rectale)* strain 16-6-I 1 FAA was affected by eugenol and vanillin, whereas ferulic acid did not have a significant effect on this isolate. This bacterium is considered a prevalent species of the human gut microbiota because it produces butyrate from acetate, with a beneficial effect on the host (Bevilacqua et al., 2016). Another sensitive strain from the Firmicutes phylum was *C. spiroforme* strain 16-6-I 21 FAA. This species has been described as responsible for enteric disease in animals such as rabbits, although no human infection has yet been reported (Uzal et al., 2018). Some members of the genus *Clostridium* are major causes of endogenous infection (Warren, Tyrrell, Citron, & Goldstein, 2006) and thus any inhibitory effect of the tested phenolic compounds could potentially help to control the colonization of these pathogens in food. However, many *Clostridium* spp. are not closely related and antimicrobial activity should be evaluated for a specific species. *E. aldenensis* (formerly *Clostridium aldenense*) strain 16-6-S 15 LS has been known to cause bacteremia under certain circumstances (Prasai et al., 2016); the tested strain in our work was found to be mainly sensitive to the exposure of ferulic acid, while eugenol and vanillin only affected growth when they were immobilized on the 10-µm amorphous silica support. The final tested Firmicutes member in this study was *M. faecis* (before classified as *Ruminococcus faecis*) strain 16-6-I 30 FAA. *M. faecis* is considered as biomarker for improving the health as a result of its capacity to produce several short-chain fatty acids (SCFAs) (Ye et al., 2021). In this study, *M. faecis* strain 16-6-I 30 FAA growth was not affected by the phenolic compounds, with the exception of some of the immobilized forms of vanillin and ferulic acid.

From the phylum Bacteroidetes, *B. caccae* (strain 32-6-I 19 NB AN) and *P. distasonis* (strain 16-6-I 5 FM) were evaluated. The growth of these strains was slightly influenced

by exposure to the phenolic compounds. The general antibiotic resistance of *Bacteroides* species has been previously reported (García-Bayona & Comstock, 2019). Both *Bacteroides* and *Parabacteroides* spp. belong to the human core intestinal microbiota, however the role of *Bacteroides* in host health in general is difficult to ascertain and likely related to the specific species; some studies consider these microbes to be beneficial, whereas other research has correlated these bacteria with a possible role in the pro-inflammatory response, mucin degradation and increased permeability of the small intestine (Bevilacqua et al., 2016). On the other hand, *P. distasonis* is considered as health-promoting potential marker as a result of its anti-inflammatory properties (Cuffaro et al., 2020).

Within the Actinobacteria phylum, the phenolic compounds had no effect on the growth of strains such as *B. faecalis* strain 16-6-I 11 FAA; similar strains potentially contribute to human health by improving gut barrier function, stimulating the host immune system, activating provitamins, and/or modulating lipid metabolism (Lee, Jenner, Low, & Lee, 2006; Ozdal et al., 2016). The phenolic compounds generally enhanced the growth of *C. aerofaciens* strain 16-6-I 3 FM, which belongs to the family *Coriobacteriaceae* that also contains some species considered as pathobionts (Gomez-Arango et al., 2018).

Strain 16-6-S 2 MRS of *E. fergusonii*, a close relative of *E. coli* (phylum Proteobacteria) was also evaluated and was found to not be notably affected by treatment with the tested phenolic compounds. Finally, *A. muciniphila* strain 3 FMU (a member of the Verrucomicrobia phylum) showed heterogeneity in its response to exposure to the free and immobilized phenolic compounds, being more sensitive to the antimicrobials in their free form, while being stimulated to grow in the presence of some forms of the immobilized presentations of the phenolics. *A. muciniphila* is an important gut symbiont for the maintenance of metabolic homeostasis which produces SCFAs, increases mucus thickness and gut barrier function; it is currently under consideration as a new probiotic (Ottman, Geerlings, Aalvink, de Vos, & Belzer, 2017).

Table 1 shows the statistical analysis of the relative AUC results by means of a multifactorial ANOVA displaying the significant influence of the factors (strain and administration form) on the impact of the phenolic compounds ($p<0.001$). The strain tested was the predominant factor that influenced the impact of eugenol and vanillin on the bacterial growth, while the form of administration predominantly influenced bacterial growth after incubation with ferulic acid.

Table 1. The F-ratio values and significance levels obtained in the multifactor ANOVA for the factors *Strain* and *Administration form* and their interaction in the relative AUC of bacterial gut isolates after exposure to the antimicrobial compounds.

	Eugenol		**Vanillin**		**Ferulic acid**	
Factor	*F-ratio*	*α*	*F-ratio*	*α*	*F-ratio*	*α*
Strain	217.73	***	46.78	***	39.35	***
Administration form	37.47	***	16.92	***	98.08	***
Strain x Administration form	60.36	***	11.34	***	16.63	***

Significance level (α): ***p-value<0.001

As well as the statistical significance of the factors, Table S5, supplementary information, presents the homogenous groups among the factors according to the phenolic compound studied. In general, the most sensitive strains were *A. rectalis* and *C. spiroforme* within Firmicutes. In contrast, the growth of strains of representative species of the phyla Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia was only slightly affected by the phenolic compounds. Immobilization of the phenolic compounds generally potentiated the inhibitory effects against tested isolates, compared to the free forms. We also tested for any possible inhibitory effect of the bare siliceous and cellulosic supports against bacterial isolates to ensure that the antimicrobial activity was only associated with the phenolic compounds. As can be seen in Figure S3, supplementary information, the growth of the microorganisms was not significantly inhibited by the non-functionalized carriers.

The inhibitory effect of antimicrobial systems based on the covalent immobilization of phenolic compounds with antimicrobial properties was previously established against surrogates of foodborne bacteria, including *Listeria innocua* and *Escherichia coli* (Ruiz-Rico et al., 2017) or food spoilage microorganisms such as *Zygosaccharomyces bailii*, among others (Ribes et al., 2019). The immobilization process preserved or even enhanced the inhibitory properties of the anchored antimicrobial compounds compared to free compounds after incubation in the presence of suspensions of the free or immobilized antimicrobials. Otherwise, the study of the inhibitory capability of antimicrobial systems against gut bacterial isolates has been limited. The differences observed in this study, compared to our previous works, are likely the result of the different characteristics of the tested strains, for example the anaerobic growth conditions required by the gut microbial isolates. A similar behavior has been previously reported. (Lee et al., 2006) studied the impact of tea aromatics and metabolites on the growth of various bacterial species representative of both commensals and pathogens under either aerobic or anaerobic conditions. Whereas the growth of *E. coli*, *L. monocytogenes* or *S. enterica* serovar Typhimurium was greatly inhibited by the phenolic compounds, the growth of strains belonging to the genera *Bacteroides*, *Bifidobacterium* and *Clostridium* was affected only minimally.

3.3. Free and immobilized phenolic compounds inhibit the growth of isolates form Firmicutes phylum

From the results shown in Figure 1, *A. rectalis* and *C. spiroforme* were chosen as the most susceptible species. The inhibitory effect of our tested phenolics on these strains is not in accordance with previous studies that focused on the influence of dietary polyphenols on the gut microbiota. Several studies reported the modulation of the gut microbiota by phenolic compounds with a reduction in abundance of pathogens such as *Clostridium perfringens* or *Clostridioides difficile* and an increase in the amounts of commensal anaerobes from the genera *Clostridium*, *Bifidobacterium* and *Eubacterium*

(Ozdal et al., 2016). In fact, some of these articles suggest the increase of *Clostridium* and *Eubacterium* spp. is related to their metabolism of phenolic compounds (Selma, Espín, & Tomás-Barberán, 2009). Therefore, the impact of the phenolic compounds tested in this study on *A. rectalis* and *C. spiroforme* was further investigated.

First, the effect of the antimicrobial compounds was studied in a concentration range between 0.25-2 mg/mL in order to establish the minimum inhibitory concentration for each strain. Figures 2 and 3 show the relative AUC of the two microorganisms, compared to the control condition, for the different concentrations of the phenolic compounds either free or immobilized on the siliceous and cellulosic supports. *A. rectalis* strain 16-6-I 1 FAA (Fig. 2) was completely inhibited by the different forms of administration of eugenol in a range of concentration of 0.5-2 mg/mL, and vanillin using a concentration between 1-2 mg/mL, while ferulic acid produced a slight effect on the strain growth only when it was immobilized ($p<0.05$). In addition to the differences with respect to the control condition, the results of the multifactor ANOVA (Table S6, supplementary information) showed a significant influence ($p<0.001$) of the form of presentation of the phenolic compound and the phenolic concentration on the growth of the bacterium. Immobilized phenolic compounds displayed higher inhibitory potential than the free antimicrobials. Among the administration forms, phenolic compounds immobilized on the amorphous silica particles were the most effective; in these forms, exposure to the phenolics resulted in the complete inhibition of *A. rectalis* with either 0.5 mg/mL of the support functionalized with eugenol or 1 mg/mL of the vanillin-functionalized carrier.

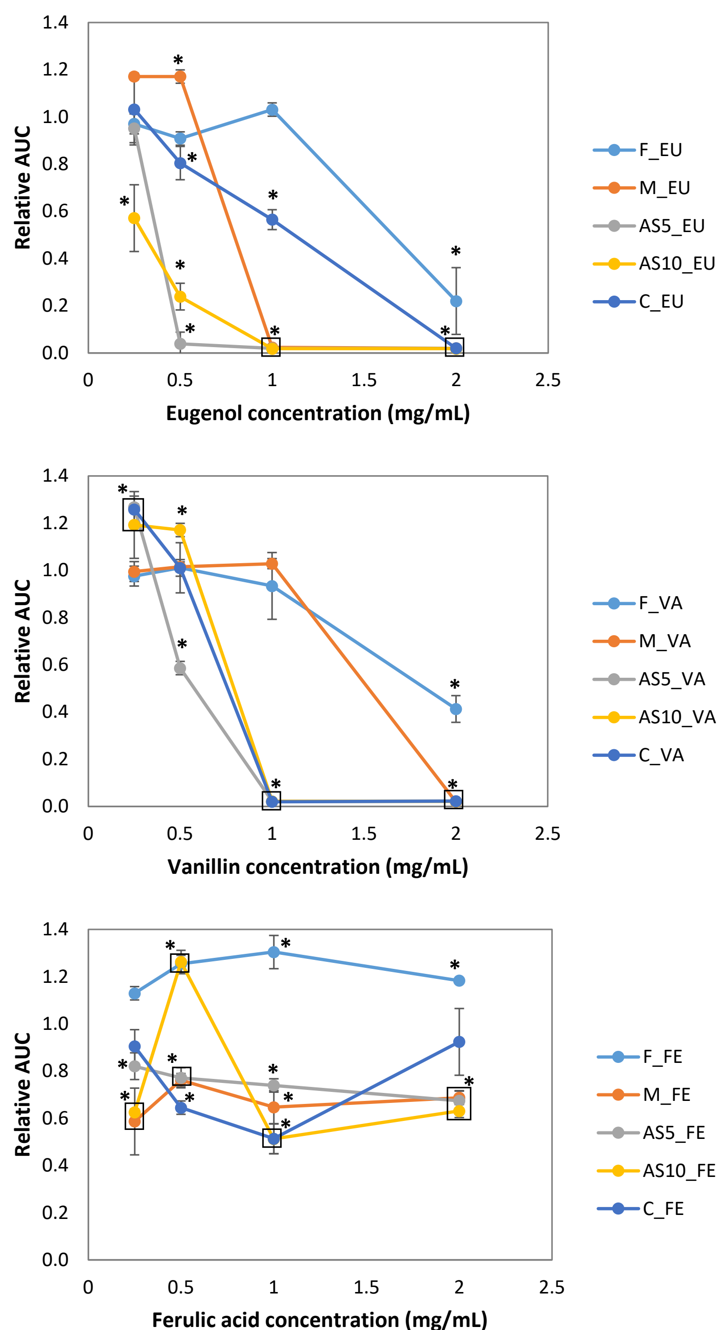

Figure 2. Relative AUC of *Agathobacter rectalis* (strain 16-6-I 1 FAA) after incubation with different concentrations of eugenol (EU), vanillin (VA) and ferulic acid (FE) administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C) (means and standard deviations, n=3). The values are relative compared with the control condition without treatment. (*) $p<0.05$ indicates significant differences compared to the control.

The growth of *C. spiroforme* strain 16-6-I 21 FAA (Fig. 3) was most sensitive to vanillin exposure, followed by exposure to eugenol>ferulic acid (*p<0.001*, Table S6, supplementary information). The different presentation forms of vanillin produced the complete inhibition of the bacterium within a range of concentrations (0.5-2 mg/mL), except for vanillin-functionalized cellulose supports that did not have any effect on the growth of the microorganism. Eugenol immobilized on silica supports was the most effective forms of administration of this phenolic compound for this microorganism, but resulted only in a partial growth inhibition using the highest concentration (2 mg/mL). Ferulic acid in its free form and immobilized on mesoporous silica particles were the only presentation forms that produced the total inhibition of *C. spiroforme* strain 16-6-I 21 FAA using 2 mg/mL of the phenolic compound. In contrast to the behavior of the phenolic compounds against *A. rectalis* strain 16-6-I 1 FAA, the free antimicrobials were generally more effective than the immobilized ones. The concentrations of antimicrobials needed to completely inhibit the growth of *C. spiroforme* strain 16-6-I 21 FAA fell within the range of previous studies with other biocides. (Hrncirova, Hudcovic, et al., 2019) evaluated the impact of synthetic food preservatives such as nitrite, sorbate or benzoate on different isolates from the human gut, observing a high susceptibility for *Clostridium tyrobutyricum* with an IC50 in the range of 0.01-1 mg/mL.

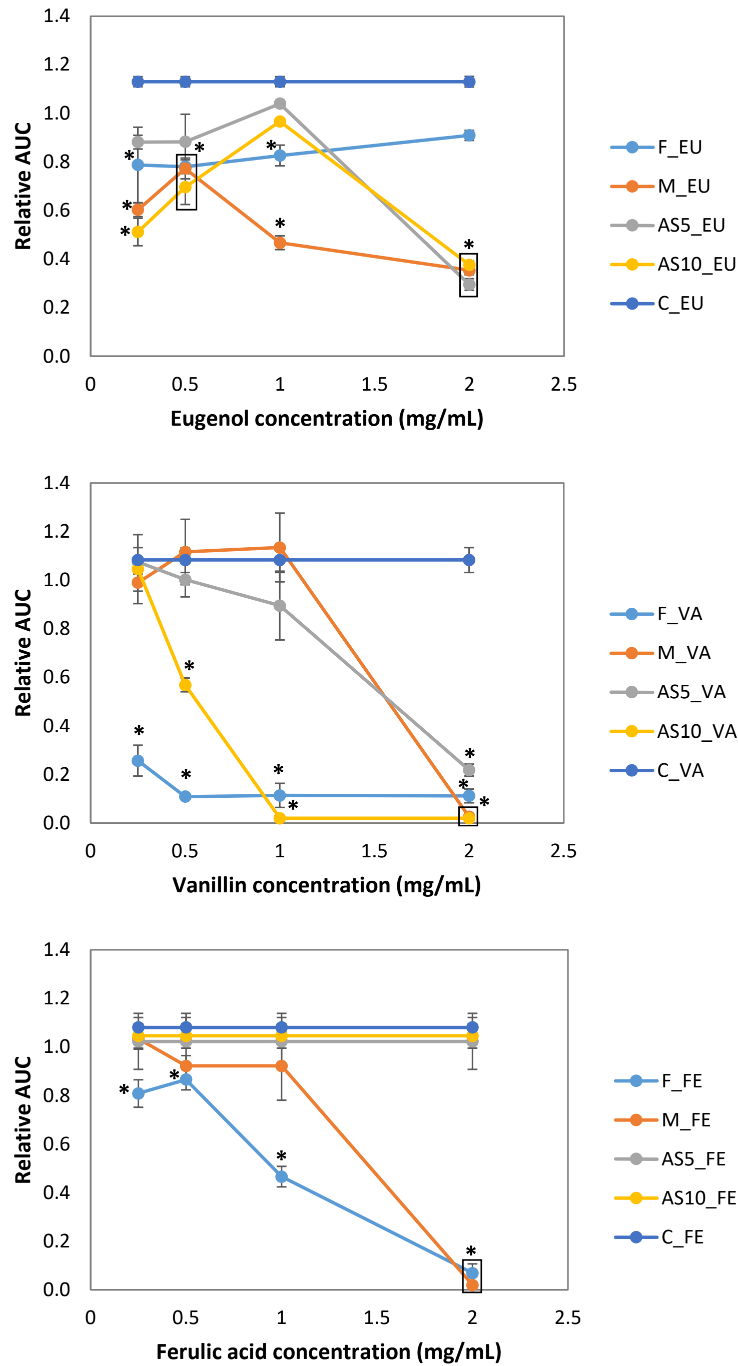

Figure 3. Relative AUC of *Clostridium spiroforme* (strain 16-6-I 21 FAA) after incubation with different concentrations of eugenol (EU), vanillin (VA) and ferulic acid (FE) administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C) (means and standard deviations, n=3). The values are relative compared with the control condition without treatment. (*) $p<0.05$ indicates significant differences compared to the control.

3.4. Impact of free and immobilized phenolic compounds is strain-specific and species-specific

Once the inhibitory effect of phenolic compounds was established on the strains defined as sensitive, the impact of these molecules on the growth of other related species of the gut microbiota was studied to assess whether results could be extrapolated more generally. Figures 4 and 5 present the effect of 2 mg/mL of free or immobilized phenolic compounds on the growth of strains of different species related to the genera *Agathobacter* and *Clostridium* present in the defined gut community, as well as different isolates of the species *A. rectalis* and *C. spiroforme* of stool samples obtained from other donors, compared to the relative AUC of the previously defined as sensitive strains (*A. rectalis* strain 16-6-I 1 FAA and *C. spiroforme* strain 16-6-I 21 FAA from donor A).

The impact of phenolic compounds in *Agathobacter*-related strains (Fig. 4) *A. rectalis* strain MPYG-30 (from Donor C) and *E. ventriosum* strain 16-6-I 47 FAA (another member of the *Lachnospiraceae* family) was significantly different from that of the reference strain (*A. rectalis* strain 16-6-I 1 FAA from donor A) ($p<0.05$). The treatment with eugenol resulted in less inhibition of *Agathobacter*-related strains than the reference strain 16-6-I 1 FAA. Vanillin was the most effective antimicrobial against these microorganisms but significant differences in susceptibility were found only after exposure to the phenolic compound immobilized on the AS5 support. With ferulic acid, *E. ventriosum* strain 16-6-I 47 FAA, and to a lesser extent *A. rectalis* strain MPYG-30 (from Donor C), showed high sensitivity to this antimicrobial in its free form or immobilized on the different carriers.

The differences observed for the *A. rectalis* strains from the different donors are consistent with inter-individual differences in gut microbiota previously reported (Catalkaya et al., 2020). Indeed, the higher susceptibility of *A. rectalis* strain MPYG-30 (from Donor C, isolated from a member of Yanomami population in a rural area) could be related to the low exposure to antibiotics, medications, and food additives in this population that may have produced the loss of the most sensitive strains from people in developed countries (Hrncirova, Machova, et al., 2019).

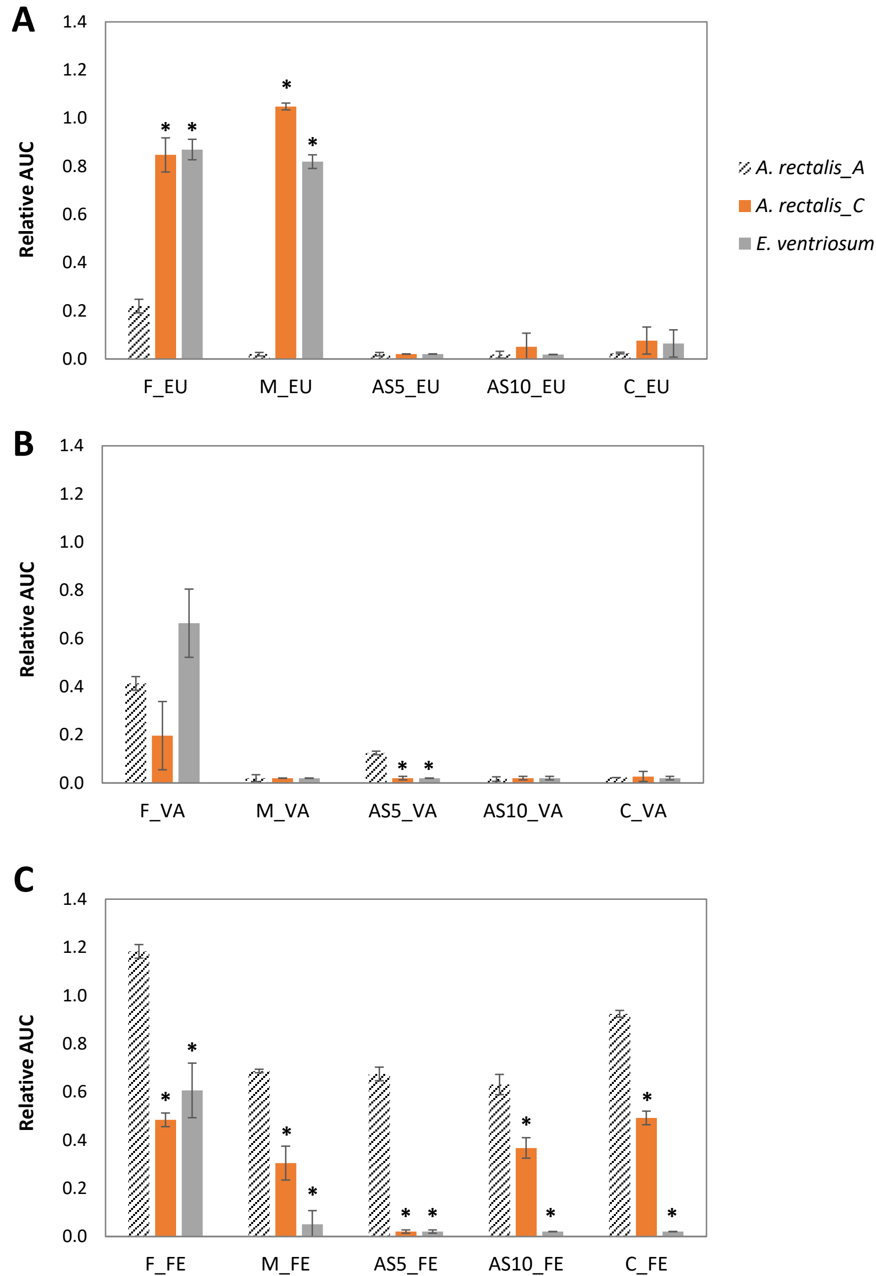

Figure 4. Relative AUC of *Agathobacter rectalis* strain MPYG-30 (from donor C) and *Eubacterium ventriosum* strain 16-6-I 47 FAA after incubation with eugenol (A), vanillin (B) and ferulic acid (C) administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C) in comparison to *A. rectalis* strain 16-6-I 1 FAA (from donor A) (means and standard deviations, n=3). Antimicrobial concentration of 2 mg/mL. (*) $p<0.05$ indicates significant differences compared to *A. rectalis* strain 16-6-I 1 FAA.

Within the genus *Clostridium* (Fig. 5), the microbial growth of the evaluated strains was statistically different than that of the reference strain (*C. spiroforme* strain 16-6-I 21 FAA from donor A) ($p<0.05$) after exposure to the phenolic compounds, resulting in a lower inhibitory effect. The results of the multifactor ANOVA (Table S7, supplementary information) showed the strong influence of the strain evaluated on the antimicrobial effect of the phenolic compounds. The diminished effect of the phenolic compounds on the other *Clostridium* species is in accordance with previous studies in which different phenolics produced a reduction of some pathogens of the genus *Clostridium* like *C. histolyticum* and enhancement of the growth of commensal *Clostridium* species (*C. cocoides-E. rectale* group) (Hidalgo et al., 2012). The species tested in this assay form part of the main members of gut microbiota population *Clostridium* cluster XIVa (*C. symbiosum*), and, to a lower extent, clusters IV, XVI (*C. innocuum*) and XVIII (*C. spiroforme*), that are considered beneficial species as a result of their recognized anti-inflammatory properties (Goldstein, Citron, & Tyrrell, 2014; Van Den Abbeele et al., 2013). Despite sharing a genus name, many *Clostridium* spp. are polyphyletic and not closely related. Consequently, considering the dissimilarities between species of a related genus and the inter-individual differences on the effect of phenolic compounds on gut microbiota, studies with a large number of species should be considered in order to extrapolate the results (Ozdal et al., 2016).

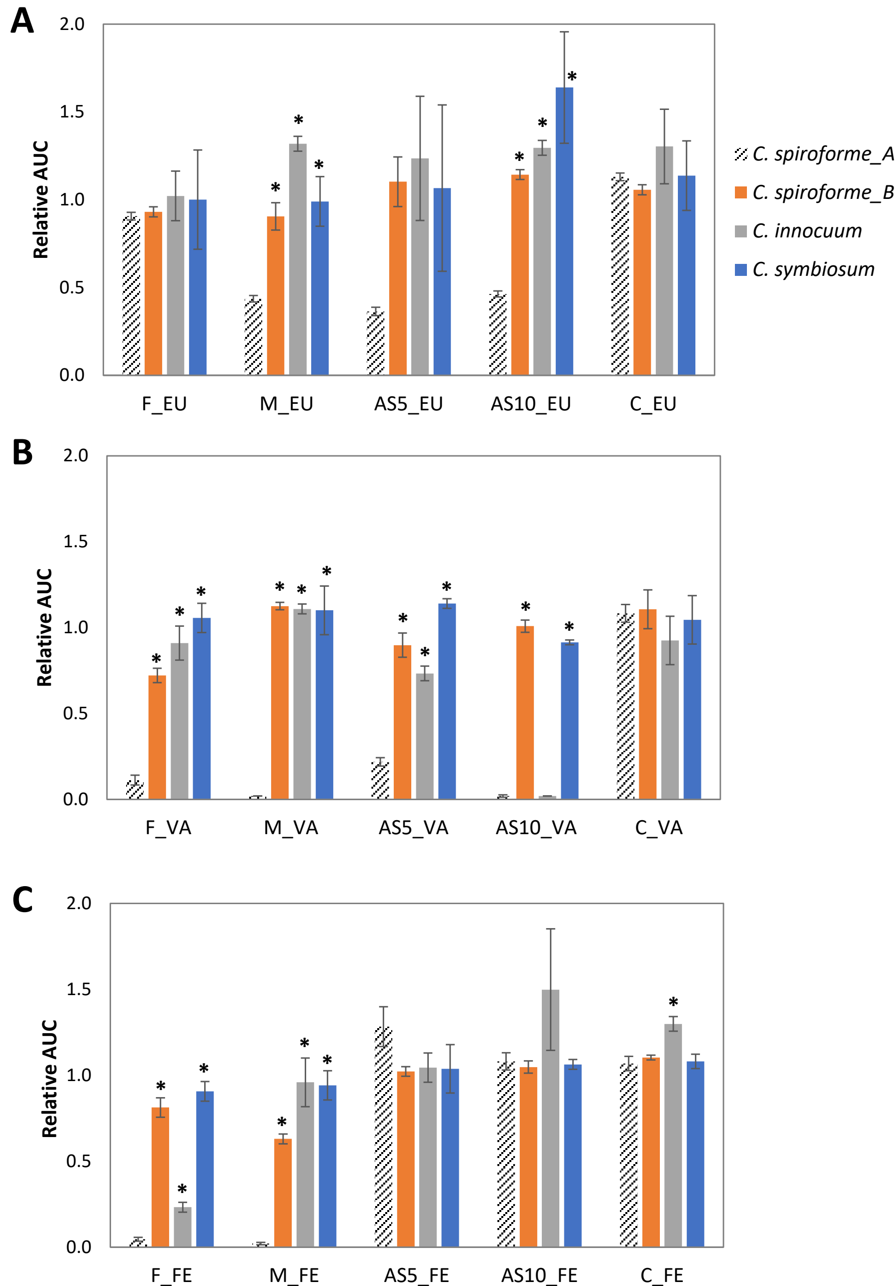

Figure 5. Relative AUC of *Clostridium spiroforme* strain 58 TSA (from donor B), *Clostridium innocuum* strain 16-6-S 16 LG and *Clostridium symbiosum* strain 16-6-S 5 FAA after incubation with eugenol (A), vanillin (B) and ferulic acid (C) administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C) in comparison to *C. spiroforme* strain 16-6-I 21 FAA (from donor A) (means and standard deviations, n=3). Antimicrobial concentration of 2 mg/mL. (*) $p<0.05$ indicates significant differences compared to *C. spiroforme* strain 16-6-I 21 FAA.

4. Conclusions

This work determined the impact of possible food preservatives based on phenolic compounds on isolates of human gut bacteria. Isolates were generally not susceptible to phenolics either free or immobilized on different carriers. Only the growth of a few strains of species belonging to the phylum Firmicutes was significantly affected by antimicrobials. Susceptibility depended on the phenolic compound, the mode of administration and the concentration tested. The high susceptibility to the phenolics tested of some Firmicutes species, which likely have an essential role in microbial metabolism, may be a concern if this results in a modification of the composition of the gut microbiota. However, the antimicrobial effect was specific to the species and even strain level, and therefore, before establishing the impact of a food preservative, the active compound needs to be evaluated for its inhibitory effects on a large number of strains to fully understand how a given compound can affect the gut microbiota. The generally slight impact of free and immobilized phenolics on the growth of isolates of commensal gut bacteria endorses their biocompatibility with the human gut microbiota that supports their use in the food industry as food preservatives or food processing aids. On the other hand, dietary supplementation with these compounds could be used to modulate the microbiota because they have the potential to enhance the growth of beneficial species, such as *Akkermansia muciniphila* or *Bifidobacterium faecale.* However, the results obtained in this study have some limitations that make it difficult to speculate on the impact of food preservatives in a real environment. This study can be considered as an initial look at the effects of food preservatives on the gut microbiota and suggest that a more comprehensive assessment of the impact of free and immobilized phenolic compounds on the composition and function of a complex ecosystem such as the gut microbiota is warranted.

CRediT authorship contribution statement

MRR: conceptualization, investigation, validation, formal analysis, writing (original draft, review & editing); SR: methodology, data curation; EAV: methodology, resources, supervision, writing (review & editing); JMB: conceptualization, funding acquisition, writing (review & editing).

Declaration of Competing Interest

Authors MRR, SR and JMB declare that they have no known competing interests that could have appeared to influence the results reported in this paper. EAV is the CSO and co-founder of NuBiyota LLC, a company that is developing human gut microbiota-based live microbial products to treat a range of indications.

Acknowledgements

The authors gratefully acknowledge the financial support from the grant RTI2018-101599-B-C21 of the project “Retos Investigación” funded by MCIN/AEI/ 10.13039/501100011033 and by “ERDF A way of making Europe”. MRR acknowledges the Generalitat Valenciana for her postdoctoral fellowship (APOSTD/2019/118). We also thank A. V. Robinson and S. J. Vancuren for providing stock of the isolated gut microbiota strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

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