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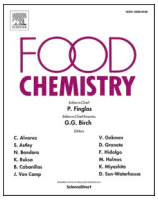

Impact of food preservatives based on immobilized phenolic compounds on an *in vitro* model of human gut microbiota

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ABSTRACT

To address concerns about the biocompatibility of novel phenolic immobilization-based food preservatives, their impact on the composition and metabonomic profile of a defined community of human gut microbiota was evaluated. Three phenolics (eugenol, vanillin and ferulic acid) presented in two forms (free or immobilized on different supports) were tested at two concentration levels (0.5 and 2 mg/mL). Free eugenol was the phenolic with the greatest impact on gut microbiota, with a remarkable increase in the abundance of *Lachnospiraceae* and *Akkermansiaceae* families. In contrast, immobilized phenolics produced an increase in the abundance of *Bacteroides* with a reduction in the ratio of Firmicutes to Bacteroidetes. The metabonomic profile was also affected by free and immobilized phenolics differently in terms of fermentation by-products and phenolic biotransformation metabolites. Thus the results suggest the importance of evaluating the impact of new compounds or materials added to food on human gut microbiota and their potential use to modulate microbiota composition.

1. Introduction

The human gut microbiota is a dynamic ecosystem that fulfills many critical roles in bodily functions, such as protection against pathogens, immune system modulation, fermentation and metabolism of nutrients, and biotransformation of xenobiotics (Claus, Guillou, & Ellero-Simatos, 2016).

Diet is one of the key environmental factors that can influence the composition, integrity and functionality of the gut microbiota as the gastrointestinal tract is the first organ exposed to elements of the diet (Catalkaya et al., 2020). Among food components, food additives are substances of the modern diet that need to be investigated because emerging evidence suggests they may alter the composition and function of gut microbiota (Hrncirova, Machova, Trckova, Krejsek, & Hrncir, 2019). The impact of different food additives such as artificial sweeteners, emulsifiers, food colorants or food preservatives on microbiota has been evaluated in recent years indicating that these substances could induce imbalances in ecosystem composition with, potentially, effects on human health (Rinninella, Cintoni, Raoul, Gasbarrini, & Mele, 2020).

Conventional food preservatives are synthetic preservatives with proven antimicrobial properties but whose safety is currently in doubt (Keeton, 2011). As an alternative to artificial preservatives, different naturally-occurring antimicrobial compounds are being proposed as substitute preservatives (Pisoschi et al., 2018). Among potential natural food preservatives, several phenolic compounds from plants have been proposed as potential preservatives given their recognized antimicrobial properties (Faustino et al., 2019; Gutiérrez-del-Río, Fernández, & Lombó, 2018). However, the direct application of these substances in food is limited by their undesirable sensory properties, inadequate solubility, and interaction with food components that constrains their antimicrobial properties and therefore, new modes of presentation such as immobilization are being developed to avoid these issues (Ruiz-Rico et al., 2017). The covalent immobilization of natural antimicrobials on inert supports allows the preservation or even the enhancement of their antimicrobial properties, preventing their spontaneous release in the medium and avoiding their absorption in the digestive tract (Ribes, Ruiz-Rico, Pérez-Esteve, Fuentes, & Barat, 2019).

The immobilization changes the behavior of the grafted antimicrobial, hindering its release in the food to which it is applied. It is expected that the antimicrobial activity, bioavailability and biocompatibility of immobilized antimicrobials will be modified after ingestion, and therefore, as well as validation of the efficacy of these food

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preservatives, their safety should also be verified prior to their introduction in food manufacture. Safety should include standard toxicity studies in cell culture (Fuentes, Ruiz-Rico, Fuentes, Barat, & Ruiz, 2021) and animal models (Verdú et al., 2020), but also the biocompatibility of the introduced compound with the gut microbiota should be assessed. Recently, our group evaluated the effect of these antimicrobial systems on individual human gut microbes and found that some isolates from the phylum Firmicutes, including *Agathobacter rectalis* and *Clostridium spiroforme*, were highly susceptible to both free and immobilized phenolics (Ruiz-Rico, Renwick, Allen-Vercoe, & Barat, 2022). However, studies examining the impact of immobilized food preservatives on human gut microbiota ecosystems are not available.

In recent years, *in vitro* microbiota models have been developed, ranging from simple batch culture to complex continuous culture or 'chemostat' systems. Using chemostats, microbial communities cultured from fresh feces can reach an equilibrium that resembles the *in vivo* distal gut community from which they originate. Being host-free systems, the gut microbiota-containing chemostats are ideal supports for studying microbial disturbances resulting from the addition of exogenous stimuli, since microbial changes can be measured independently of any simultaneous effect on the host. Other advantages of a chemostat model include the capacity for tight control, reduced expense (compared to *in vivo* models) and ease of sampling as often as necessary without ethical restrictions (Santiago-Rodriguez et al., 2015). The use of *in vitro* microbiota models together with molecular approaches such as metagenomics or metabonomics should prove to be useful for revealing the composition and the metabolic kinetics of a given ecosystem in response to different environmental stimuli (Hervert-Hernández & Goñi, 2011).

In this work, we aimed 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) using an *in vitro* model of the human gut microbiota. The effect of phenolic compounds on a gut microbiota ecosystem was studied by exposing the defined community in batch anaerobic fermentation to antimicrobials, monitoring the disturbances in the composition and viability of the human microbiota and its metabolic profile.

2. Materials and methods

2.1. Synthesis and characterization of siliceous and cellulosic supports functionalized with phenolic compounds

The phenolic compounds eugenol (99 % w/w, Sigma-Aldrich, Madrid, Spain), vanillin (99 % w/w, Ernesto Ventós S.A., Barcelona, Spain) and *trans*-ferulic acid (99 % w/w, Sigma-Aldrich, Madrid, Spain) were chosen as natural antimicrobials to create the phenolic-functionalized materials. These phenolic compounds have a similar molecular structure but the slight differences influence their antimicrobial properties and the immobilization approach (Ruiz-Rico et al., 2017) that can modify the impact on the gut microbiota ecosystem (Ruiz-Rico et al., 2022). The inert supports used as the immobilization carriers consisted of synthesized mesoporous silica microparticles MCM-41, commercial silica gel microparticles of mean particle size of 5 μm (Silysiamont, Milano, Italy) and 10 μm (Sigma-Aldrich, Madrid, Spain), and microcrystalline cellulose particles (Sigma-Aldrich, Madrid, Spain). MCM-41 is a mesoporous material with high stability and biocompatibility that has been proposed for a wide range of new biotechnological applications (Aznar et al., 2016). Commercial microparticles used in this study are considered GRAS (generally recognised as safe) materials and authorised as food additives (amorphous silica microparticles, E-551; and cellulose microparticles, E-460) (García-Ríos, Ruiz-Rico, Guillamón, Pérez-Esteve, & Barat, 2018).

The synthesis of MCM-41mesoporous silica microparticles was carried out following the 'atrane route' following the methodology previously described by (Fuentes, Ruiz-Rico, Fuentes, Ruiz, & Barat, 2020) (see Supplementary Information for details). The covalent immobilization of the phenolic compounds on the carriers' surface was performed by surface salinization according to the methodology described in (Ruiz-Rico, García-Ríos, Barat, & Guillamón, 2021) using (3-aminopropyl) triethoxysilane (Sigma-Aldrich, Madrid, Spain) as organosilane for the grafting of the organic molecules through the amine functional moiety (see Supplementary Information for details).

The characterization of non-functionalized and phenolic-functionalized supports were carried out by standard techniques including dynamic light scattering (DLS), zeta potential and elemental analysis. Particle size distribution ($d_{0.5}$) 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 (ζ) was determined by zeta potential analysis using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The electrophoretic mobility measurements were converted into ζ 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 and the analyses were conducted in triplicate. Degree of functionalization (α) was established by elemental analysis for C, H and N in a CHNOS Vario EL III model (Elemental Analyses System GMHB, Germany). Results were statistically analyzed by an analysis of variance (ANOVA) using Statgraphics 18 (Statpoint Technologies, Inc., Warrenton, USA).

2.2. Defined microbial ecosystem

A 124-strain, 69-species defined bacterial community from a collection of previously-isolated strains originally derived from a stool sample of a healthy donor was used as a microbiota model (Petrof et al., 2013). This microbial community contains a variety of bacterial species representative of microbes native to the common human gut, which can be grown as an ecosystem and used for exposure studies (Dudefoi, Moniz, Allen-Vercoe, Ropers, & Walker, 2017).

2.3. Chemostat

The ecosystem was cultured *in vitro* within a continuously feeding bioreactor, to simulate the human intestine (see Scheme S1, Supplementary Information, for details). This system recapitulates the conditions that mimic the human colon and allows for periodic sampling to assess the influence of exposure to various environmental agents such as nutrients, additives or xenobiotics (McDonald et al., 2013). Community stocks previously prepared by combining equivalent volumes of each ecosystem strain and stored frozen were used as initial biomass for the chemostat.

Stock samples were brought from −80 °C to room temperature to thaw under anaerobic conditions before being added to degassed medium within two 500-mL Multifors bioreactor vessels (Infors HT, Switzerland). The growth medium contains a mixture of insoluble and soluble starches, vitamins, trace elements and porcine gastric mucin (see Table S1, Supplementary Information, for details). The community was maintained 24 h in batch culture within the chemostat to allow an increase in biomass, and then the bioreactor was run under the following conditions to mimic the physiological conditions found within the human colon: (i) 37 °C, (ii) pH 7.0, (iii) retention time of 24 h (500 mL of feed added per vessel per day at a constant rate while maintaining volume), and (iv) anaerobic conditions through bubbling N_2. The vessels were kept at pH 6.9–7.0 by the automatic addition of acid (5 % (v/v) HCl) or base (5 % (w/v) NaOH) (McDonald et al., 2013). The bioreactor was maintained for 14 days prior to harvest to achieve steady-state equilibrium. Three replicates of 2-mL samples were taken daily to analyze the evolution of the ecosystem. The samples were stored at −80 °C until use.

From the ecosystem obtained, exposure to antimicrobial systems was carried out by incubating the ecosystem in the presence of free or

immobilized antimicrobials in discontinuous culture (Dudefoi et al., 2017). After reaching steady-state equilibrium, 50-mL samples were harvested from the vessels every alternate day for 15 days. The ecosystem suspension was aliquoted into 5-mL samples in sterile conical tubes in the anaerobic chamber. Ecosystem samples were exposed to antimicrobials by adding free or immobilized compounds to achieve final concentrations of 0.5 and 2 mg/mL. For the phenolic compounds in their free form, stock concentrated solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, USA). The range of concentrations tested was chosen according to previous *in vitro* studies with free and immobilized phenolic compounds (Ruiz-Rico et al., 2017; Verdú et al., 2020).

Then, the inoculated tubes were sealed and transferred to a sealed container with anaerobic gas packs and incubated for 48 h at 37 °C under orbital shaking. Untreated samples and samples exposed to the solvent (DMSO) and bare supports were included as control samples. All conditions were carried out in triplicate. After exposure, 1.5-mL samples (x3) were collected and stored at −80 °C until use. The evaluation of the impact of antimicrobials on the microbiota was carried out by monitoring the composition, viability, and metabolic profile of the ecosystem.

2.4. 16S rRNA gene compositional profiling

Dynamic changes in the gut microbial community were analyzed by 16S rRNA gene sequencing. In order to profile only living bacteria, propidium monoazide (PMA) pretreatment was performed with the exposed chemostat samples to exclude DNA from bacteria with compromised membrane structure to subsequent amplification (Stinson, Keelan, & Payne, 2019). For that, 1.25 μL of 20 mM PMA dye (Biotium, Fremont, USA) was added to the cell pellet of 1.5-mL chemostat samples suspended in 500 μL of PBS to a final concentration of 50 μM PMA. The samples were covered with aluminum foil and incubated at room temperature for 15 min with occasional vortexing. The samples were then photolyzed under an LED light to cross-link PMA to DNA for 15 min with vortexing every 5 min to ensure all parts of the sample were exposed to light. The samples were then centrifuged and the pellet was resuspended in 200 μL of PBS. QIAamp PowerFecal Pro DNA Kit (Qiagen Inc., Germantown, USA) was utilized according to the manufacturer's directions to extract gDNA from the PMA-treated samples. 16S rRNA gene libraries were prepped with 400 ng of Nextera XT Index V2 sequences (Illumina, Inc., San Diego, USA) plus standard V4 region primers (515F: GTGYCAGCMGCCGCGGTAA and 806R: GGACTACNVGGGTWTCTAAT) and 2 μL of gDNA template in Invitrogen Platinum PCR SuperMix High Fidelity (Life Technologies, Burlington, Canada) as a one-step PCR amplification. Cycler conditions included an initial melting step of 94 °C for 2 min, followed by 50 cycles of 94 °C for 30 s, annealing temperature for 30 s, and 68 °C for 30 s, with a final extension step of 68 °C for 5 min. The annealing temperature was comprised of a 0.5 °C increment touchdown starting at 65 °C for 30 cycles, followed by 20 cycles at 55 °C. PCR products were purified using an Invitrogen PureLink PCR purification kit (Life Technologies, Burlington, Canada) according to the manufacturer's directions. Subsequent normalization and Illumina MiSeq sequencing were carried out at the Advanced Analysis Center located at the University of Guelph, Ontario, Canada. Sequencing data were processed using the R package DADA2 (Callahan et al., 2016), with classification to the genus level by the SILVA database, version 132 (https://benjjneb.github.io/dada2/training.html). Amplicon sequencing variants (ASVs) were next classified to the species level by first identifying the top hits of NCBI BLAST searches (https://blast.ncbi.nlm.nih.gov) via best match as determined by percentage identity and E-value of BLAST alignment, followed by cross-referencing with the known members of the defined microbial communities to determine the correct identification. ASVs that classified to redundant species were amalgamated so that each ASV was attributed to a unique species, and those that did not represent 0.01 % total abundance in at least one sample were removed. Similarly, a set of ASVs at the genus level was created by amalgamating ASVs that were classified to the same genus (Oliphant et al., 2020).

2.5. 1D ^{1}H NMR metabonomics

The metabolic profile of the ecosystem was characterized by 1D ^{1}H NMR spectroscopy. Sterile-filtered cell-free media from defined microbial ecosystems were prepared to characterize small-molecule metabolites of the microbiota. 540 μL of the samples were combined with 60 μL of the internal standard 5 mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d6) in deuterated water (D_2O), containing 0.1 % sodium azide (Chenomx Inc., Edmonton, Canada). Samples were then transferred to Wilmad 5-mm glass NMR tubes and stored at 4 °C overnight. Samples were allowed to return to room temperature before scanning on a Bruker Avance 600 MHz spectrometer, housed at the NMR Centre (Advanced Analysis Center, University of Guelph). All NMR spectra were obtained using the first increment of a 1D METNOESY pulse sequence with a mixing time of 0.1 s, 3.0 s acquisition time, and 2.0 s relaxation delay for adequate water suppression. Spectra were acquired with 32 scans. Sample pH was measured immediately after scanning using colorimetric pH indicator strips (Whatman) (Ganobis et al., 2020).

Spectra files were processed in their entirety using Chenomx NMR Suite 8.5 (Chenomx Inc., Canada). Briefly, all spectra were phase and baseline corrected using the Chenomx automatic tools with subsequent manual adjustment when required. Untargeted and targeted profiling was used to identify and quantify compounds in the pre-processed NMR spectra. Compounds were fit to spectra based on their signatures and properties obtained from the Chenomx software database, as calibrated to the internal standard, DSS. Metabolite concentrations were generated from the area of the projected signal after it was fit to the peak centers during identification.

2.6. Statistical analysis

The impact of treatment with free or immobilized antimicrobials on compositional profiling and metabonomics was evaluated using MicrobiomeAnalyst (Chong, Liu, Zhou, & Xia, 2020) and MetaboAnalyst (Chong & Xia, 2018), respectively.

For compositional data, relative read counts (57–70 ASVs and 40–44 ASVs after data filtering) from replicates (27 samples) of the 6 experimental groups (control samples, samples treated with the free antimicrobials and samples treated with the antimicrobial immobilized on mesoporous silica, 5 μm-amorphous silica 10 μm-amorphous silica and cellulose) were processed including data filtering to remove low abundance features (low count filter with a minimum count of 4 and prevalence of 20 % in samples and low variance filter based on 10 % interquantile range), and normalization of samples by centered log ratio data transformation. Alpha-diversity analysis was used to measure the diversity present within the experimental groups for the Chao1 richness index, and significant differences were evaluated using *t*-tests. Hierarchical cluster analysis in the form of a dendrogram was performed by using Jaccard index as parameter to measure distance between samples and Ward's linkage as parameter of clustering algorithms to minimize the sum of squares of clusters. Phylogenetic composition of community at genus level using stacked bar of relative abundance was performed to visualize the taxonomic composition of community untreated or treated with the antimicrobials through direct quantitative comparison of abundances. Univariate analysis was used to identify differentially abundant features in the community after treatment with the antimicrobials at genus level. The statistical significance of this analysis was determined in comparison to control group using a one-way ANOVA *t*-test.

In the case of the metabonomics data set, concentrations of metabolites (45 compounds) from replicates (24 samples) were processed for sample normalization by auto scaling (mean-centered and divided by the standard deviation of each variable). Principal Component Analysis

(PCA) of the 3 antimicrobials and the 2 level of concentrations were performed to reduce the number of variables and to detect patterns in the relationships between variables, since data obtained from metabonomics were highly correlated. After multivariate statistics, one-way ANOVA and post-hoc analysis by Fisher's LSD were used to provide a preliminary overwiew about compounds significantly affected by the antimicrobial treatment. Then, statistical significance among experimental groups was determined in comparison to control group using *t*-tests.

3. Results and discussion

3.1. Characterization of phenolic-functionalized supports

The covalent immobilization of phenolic compounds (eugenol (EU), vanillin (VA) and ferulic acid (FE)) on the surface of different carriers (MCM-41 (M), 5-μm amorphous silica (AS5), 10-μm amorphous silica (AS10) and microcrystalline cellulose particles (C)) resulted in the 12 phenolic-functionalized supports with previously proven antimicrobial properties (Ruiz-Rico et al., 2021; 2017). These supports joint to the non-functionalized materials were characterized to determine their particle size, surface charge and degree of functionalization (Table S2, Supplementary Information). The particle size results ($d_{0.5}$) showed that carriers are microparticles of mean size in a wide range from the smallest support of 0.67 ± 0.08 μm of the M particles to the largest support of 8.0 ± 0.5 μm of the AS10 particles and medium-size supports of 3.6 ± 0.4 and 4.6 ± 0.4 μm of the AS5 and C particles, respectively. The mean size of the carriers is in accordance with previous studies (Fuentes et al., 2020) and with the technical information of the specification sheet of the commercial materials. The immobilization of the phenolic compounds on the surface of the supports did not have a significant effect on the particle size of the carriers.

The zeta potential results were significantly affected by the functionalization. The non-functionalized particles exhibited negative zeta potential values because deprotonated hydroxyl groups are present on the surface of cellulosic and siliceous materials, while the supports functionalized with phenolic compounds displayed positive values in most cases because of the grafting of the phenolic-organosilane derivatives. In addition, elemental analysis of the phenolic-functionalized silica supports showed the degree of functionalization of the carriers. Both parameters confirm the immobilization of the phenolic compounds on the surface of the supports.

3.2. Impact of free and immobilized phenolic compounds on defined gut microbiota ecosystem composition

The impact of phenolic compounds on the gut microbiota was evaluated using a chemostat as an *in vitro* model of the colon that was inoculated with a defined community of a stool sample from a healthy donor. After the establishment of the microbial ecosystem, the chemostat samples were separately treated with the phenolic compounds at two concentration levels (0.5 and 2 mg/mL) in their different modes of presentation. Subsequently, a compositional analysis was performed by profiling sequenced 16S rRNA genes to elucidate which microorganisms were influenced under each tested condition. Compositional analysis results from the two concentration levels were merged for better visualization of the impact of the different modes of presentation of the antimicrobials.

Treatment with eugenol produced different changes in microbial diversity depending on the mode of presentation. Fig. 1A shows the significant changes in richness in the community profile for the eugenol-treated samples, as assessed by alpha-diversity analysis ($p < 0.05$). Exposure to eugenol in its free form, significantly reduced the number of taxa present in the samples compared to the control group, probably as a result of partial and differential inhibition of taxa within the microbial population, which in turn might potentially contribute to imbalances in ecosystem composition (Hrncirova et al., 2019). Dendrogram analysis (Fig. 1B) based on Jaccard distance metrics, which is a qualitative measure of community dissimilarity, showed the separation between control samples and eugenol-treated samples at the genus level, revealing the significant effect of the phenolic compound on microbiota composition.

The phylogenetic composition at the genus level of the non-treated samples (control) and the samples treated with the different forms of

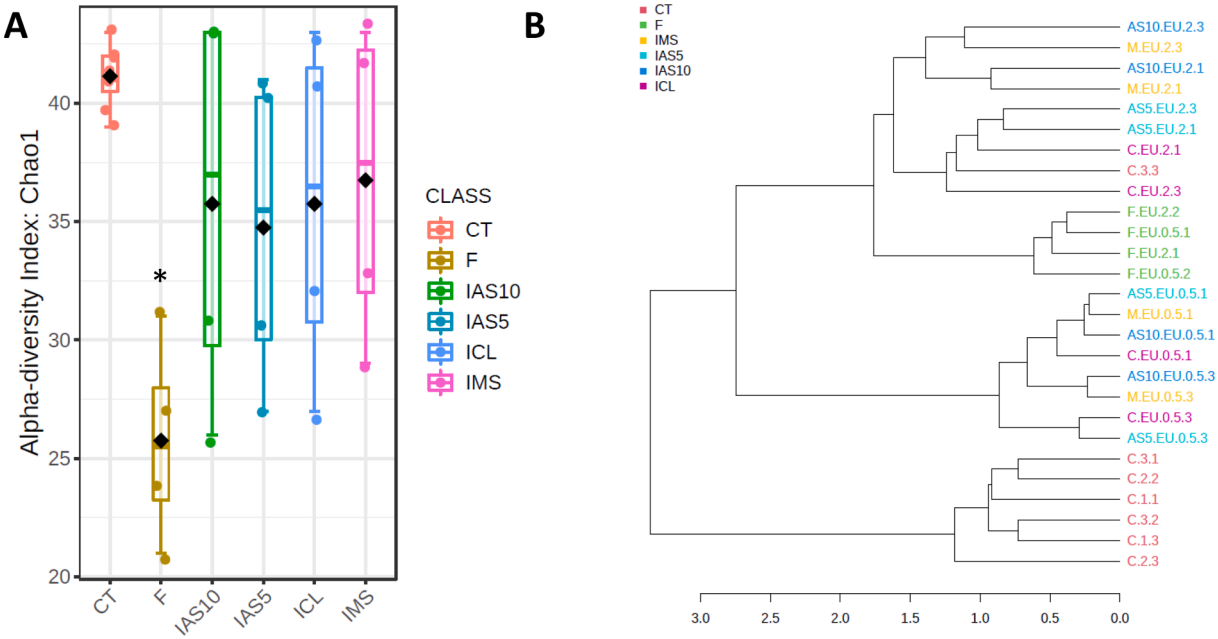

Fig. 1. (A) Boxplot of alpha-diversity analysis based on Chao1 richness of defined gut microbiota community after treatment with eugenol. (*) $p < 0.05$, *t*-test. (B) Dendrogram of defined gut microbiota community based on Jaccard distance at the genus level. CT: control samples, F: samples treated with the free antimicrobial, IAS10: samples treated with the antimicrobial immobilized on 10 μm-amorphous silica, IAS5: samples treated with the antimicrobial immobilized on 5 μm-amorphous silica, ICL: samples treated with the antimicrobial immobilized on cellulose, IMS: samples treated with the antimicrobial immobilized on mesoporous silica.

presentations of eugenol were then analyzed (Fig. 2A). The control group consisted mainly of *Acidaminococcus* (41.7 %), *Bacteroides* (17.8 %), *Akkermansia* (9.6 %), *Eubacterium* (7.8 %), and *Ruthenibacterium* (6.9 %) genera. These results showed a modification in the composition of the ecosystem after harvesting the chemostat in which the community was mainly defined by the genera *Flavonifractor*, *Bacteroides*, *Akkermansia* and *Hungatella* (data not shown). These changes could be related to the 48-h incubation period of the samples required for exposure to the antimicrobials after harvesting. During this period, the media and waste were not replaced and therefore lower amount of nutrients and higher concentrations of fermentative metabolites were found in the media (significant change in pH and metabolite profile, see Section 3.3 for more details) that may provoke changes in the composition and diversity of the gut ecosystem.

Treatment with eugenol produced different changes in the composition depending on the mode of presentation (Fig. 2A). Samples treated with free eugenol consisted mainly of *Akkermansia* (47.6 %) and *Hungatella* (44.9 %) genera. The different forms of immobilized eugenol changed the ecosystem composition being the most abundant genera *Bacteroides* (IAS10_73.6 %, IAS5_65.8 %, ICL_50.0 % and IMS_75.7 %) and *Akkermansia* (IAS10_11.7 %, IAS5_20.8 %, ICL_29.9 % and IMS_7.8 %). Therefore, immobilized eugenol reduced the ratio of Firmicutes to Bacteriodetes while free eugenol increased the abundance of Firmicutes and Verrucomicrobia. A lower ratio of Firmicutes to Bacteroidetes after exposure to phenolics was recently reported (Dou, Chen, Huang, & Fu, 2022); this study focused on the bioaccesibility and bioactivity of blackberry after digestion.

In order to screen the key genera that respond to eugenol treatment, univariate-statistical analyses (ANOVA/*t*-test) were performed. The comparison of the relative abundances of the genera significantly affected ($p < 0.05$) by eugenol treatment are presented in Fig. 2B. The abundance of some of the predominant genera in the ecosystem, *Bacteroides* and *Ruthenibacterium*, were reduced by free eugenol, while the genera *Anaerostignum*, *Hungatella* and *Lachnoclostridium* increased significantly for these samples. (Wlodarska, Willing, Bravo, & Finlay, 2015) evaluated the immunomodulatory function of eugenol on the mucosal immune response of mice gut, affirming that eugenol supplementation promoted an increase in abundance of species from the class Clostridia, including *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae* 1 and *Peptostreptococcaceae* families, resulting in mucus layer thickening. Similarly, this study has shown that treatment with free eugenol resulted in a strong increase in abundance of the *Lachnospiraceae* family (*Lachnoclostridium, Hungatella* and *Anaerostignum* genera), and to a lower extent in *Akkermansiaceae*. The increase of *Akkermansia* abundance has previously been related to the administration of polyphenols from diet or treatment with medicinal herbs resulting in a beneficial effect on host metabolism (Derrien, Belzer, & de Vos, 2017). The treatment with 0.5 mg/mL of free eugenol produced a stark increase in the level of the genus *Akkermansia*, while the effect of the high concentration immobilized eugenol was more discrete. However, without absolute abundance values, it is not clear whether *Akkermansia* increased in number or remained stable while the other genera of the ecosystem were severely impacted.

The treatment with immobilized eugenol produced an increase in abundance of *Bacteriodes* and *Parabacteroides* (Bacteroidetes phylum) with a reduction in the abundance of the other significant genera. Both genera are considered the predominant bacteria within the human intestinal tract with beneficial effects on the host mainly due to the capacity of producing SCFA metabolites (Lv et al., 2017).

In all of the eugenol-treated samples, a reduction of the genera *Acidaminococcus* and *Eubacterium* (both of which belong to the Firmicutes phylum), and the genus *Sutterella* (which belongs to the Proteobacteria phylum), can be observed regardless of the administration form, compared to the control samples.

Regarding the results of vanillin treatment, ecosystem richness was more affected by the exposure to vanillin-functionalized supports than by free form in accordance with alpha-diversity analysis ($p < 0.05$) (Fig. 3A). However, dendrogram analysis (Fig. 3B) could not demonstrate a clear separation of the groups of samples. The phylogenetic composition of the gut community exposed to vanillin was further examined (Fig. 4A), showing that immobilized vanillin resulted in a significant increase in Bacteroidetes (*Bacteriodes* genus with a relative abundance of 69.6 % (IAS10), 65.8 % (IAS5), 27.4 % (ICL) and 73.0 % (IMS)) and Verrucomicrobia (*Akkermansia* genus with a relative abundance of 13.5 % (IAS10), 21.3 % (IAS5) and 40.2 % (ICL)) and a decrease of Firmicutes (mainly *Acidaminococcus* genus). In this case, the free phenolic compound did not cause important changes to the microbiota composition (*Acidaminococcus* 30.7 %, *Bacteroides* 23.8 %, *Akkermansia* 6.3 %, *Eubacterium* 11.3 % and *Ruthenibacterium* 9.1 %).

The relative abundances across the experimental groups were compared and significant affected genera ($p < 0.05$) are shown in Fig. 4B. Immobilized vanillin forms showed higher effect on the relative

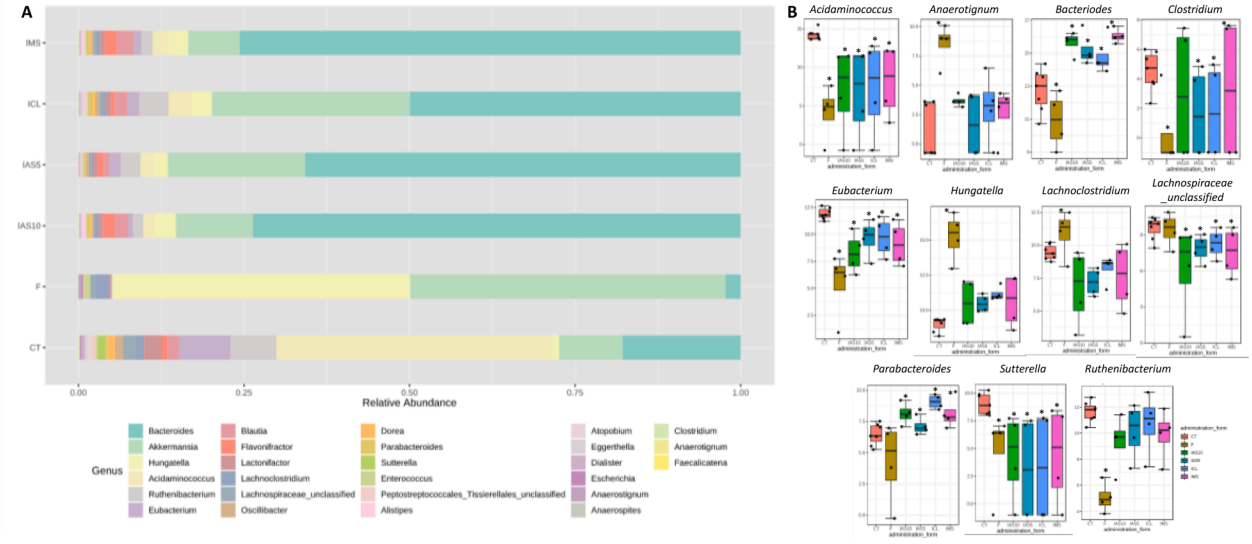

Fig. 2. (A) Histogram of relative abundance at the genus level for the non-treated and eugenol-treated microbiota samples. (B) Log-transformed counts of genera significantly different after exposure to eugenol in its free form (F) or immobilized in 10 μm-amorphous silica particles (IAS10), 5 μm-amorphous silica particles (IAS5), cellulose particles (ICL) and mesoporous silica particles (IMS), in comparison to control samples (CT). (*) $p < 0.05$ indicates significant differences compared to the control.

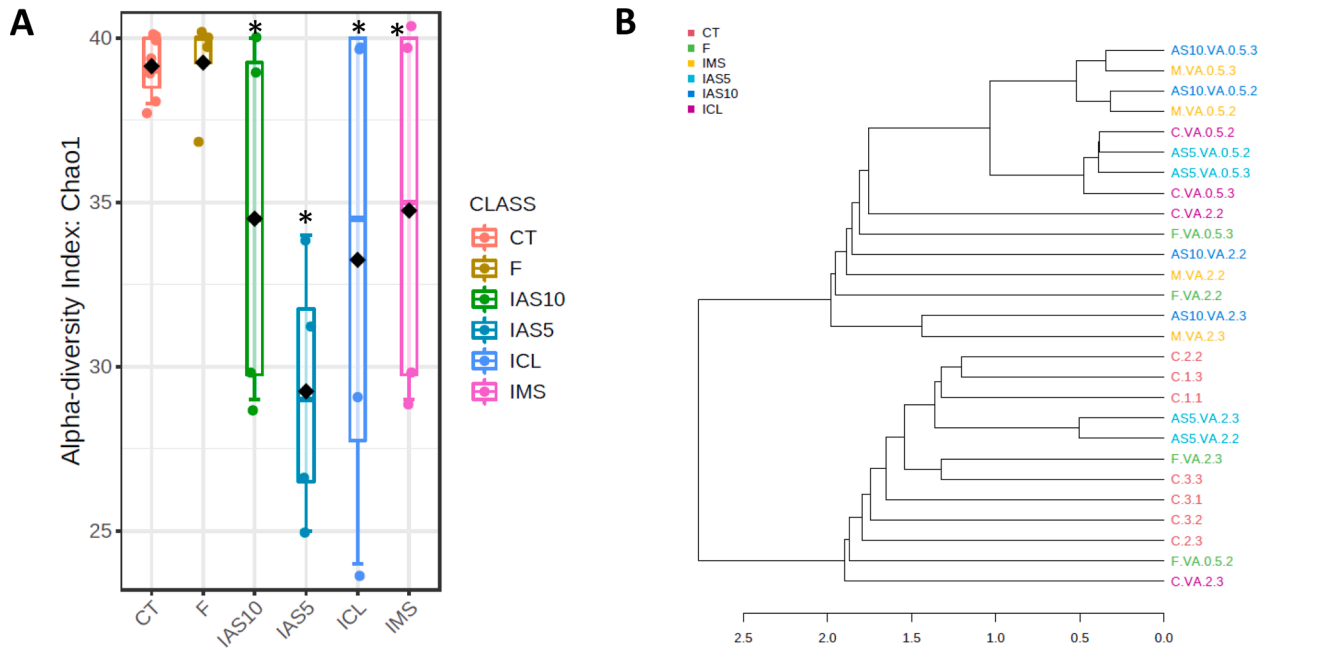

Fig. 3. (A) Boxplot of alpha-diversity analysis based on Chao1 richness of defined gut microbiota community after treatment with vanillin. (*) $p < 0.05$, *t*-test. (B) Dendrogram of defined gut microbiota community based on Jaccard distance at the genus level. CT: control samples, F: samples treated with the free antimicrobial, IAS10: samples treated with the antimicrobial immobilized on 10 μm-amorphous silica, IAS5: samples treated with the antimicrobial immobilized on 5 μm-amorphous silica, ICL: samples treated with the antimicrobial immobilized on cellulose, IMS: samples treated with the antimicrobial immobilized on mesoporous silica.

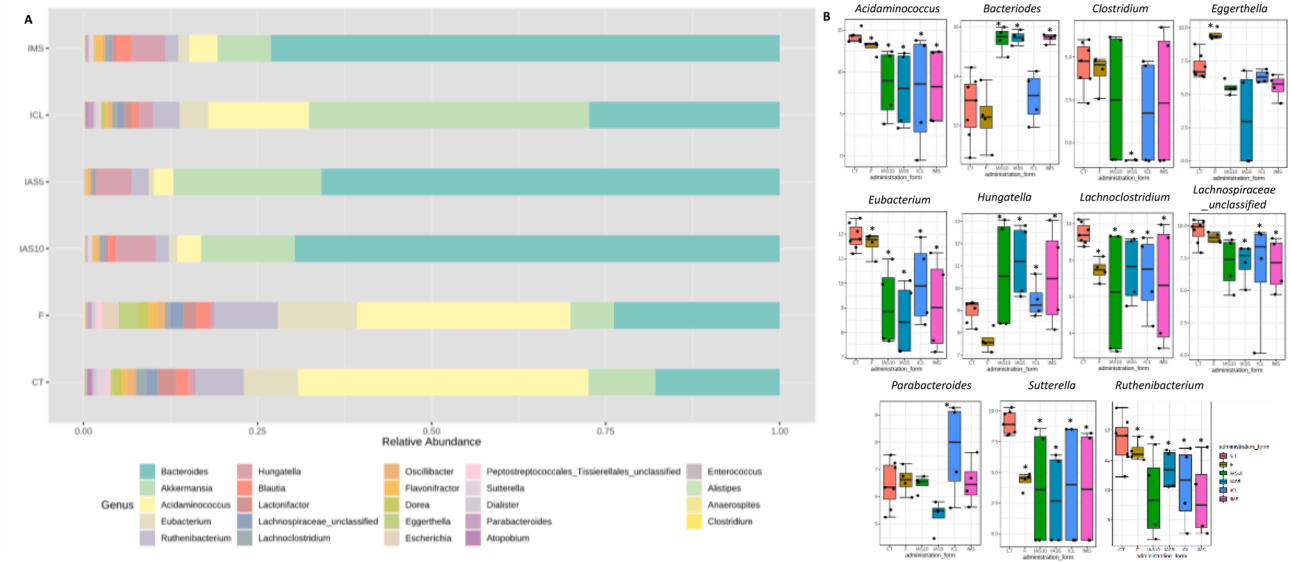

Fig. 4. (A) Histogram of relative abundance at the genus level for the non-treated and vanillin-treated microbiota samples. (B) Log-transformed counts of genera significantly different after exposure to vanillin in its free form (F) or immobilized in 10 μm-amorphous silica particles (IAS10), 5 μm-amorphous silica particles (IAS5), cellulose particles (ICL) and mesoporous silica particles (IMS), in comparison to control samples (CT). (*) $p < 0.05$ indicates significant differences compared to the control.

abundance of the different genera compared to the free vanillin. Univariate statistical analysis (ANOVA/*t*-test) revealed the reduction of the genera *Acidaminococcus*, *Eubacterium*, *Lachnoclostridium*, *Clostridium, Ruthenibacterium*, *Lachnospiraceae*_unclassified (all Firmicutes), and *Sutterella* (Proteobacteria) for the vanillin-treated samples compared to controls. In contrast, the treatment with immobilized vanillin resulted in an increase of the genera *Bacteroides, Parabacteroides* (Bacteroidetes) and *Hungatella* (Firmicutes). Free vanillin exposure resulted in slight reduction of the genera *Acidaminococcus, Eubacterium, Lachnoclostridium, Ruthenibacterium* and *Sutterella*, with an increase in the relative abundance of *Eggerthella*.

Regarding the results of ferulic acid treatment, alpha diversity analysis showed non-significant differences ($p > 0.05$) (Fig. S1A, supplementary information). However, dendrogram analysis separated samples into three groups: control samples, samples treated with 0.5 mg/mL of ferulic acid and samples treated with 2 mg/mL of ferulic acid (Fig. S1B). Similarly to the other compounds evaluated, exposure to ferulic acid resulted in an increase in the abundance of *Bacteroides* and *Akkermansia* and a reduction in *Acidaminococcus* and *Eubacterium* (Fig. S2, supplementary information).

Overall, the tested phenolic compounds in their different modes of presentation had a significant impact on the composition of the defined

community. Free eugenol significantly affected the gut microbiota composition favoring an increase in the abundance of the phylum Firmicutes with a reduction in Bacteroidetes and Proteobacteria, whereas free vanillin and ferulic acid produced only a slight impact. The increase in the abundance of Firmicutes by eugenol corresponded to an increase in the proportion of genera from the *Lachnospiraceae* family. Members of this family form part of the *Clostridium* cluster XIVa, and are major contributors to the production of SCFAs which, in turn, are known to help prevent inflammatory processes (Ma & Chen, 2020). Despite the effect of the free phenolic compounds on the tested microbiota ecosystem, it is important to appreciate that these phenolic substances may be differentially bioavailable in different individuals according to their absorbance profiles, with the likelihood that they may be easily absorbed in the small intestine and the concentration that reaches the colon would be minimal (Ozdal et al., 2016). It is also important to be aware that, regardless of changes in the composition of the microbiota, phenolic compounds in the colon may have direct interactions with intestinal tissue resulting in beneficial health effects such as the stimulation of mucus secretion, (Wlodarska et al., 2015).

In contrast, the use of phenolic compounds immobilized on inert supports modifies their performance in the gastrointestinal tract (preventing their absorption) and their impact on the gut microbiota. An example of these changes is that the exposure to the immobilized antimicrobials produced a clear increase in the abundance of the *Bacteroides* genus (mainly *Bacteroides fragilis* species). Previous *in vitro* and *in vivo* studies, which focused on the effect of polyphenol supplementation, showed no influence on the abundance of *Bacteroides* in the human gut microbiota as a result of polyphenol treatment (Ma & Chen, 2020), while a more recent study reported the reduction of the ratio of Firmicutes to Bacteroidetes after *in vitro* exposure to phenolics from blackberry fruit; this ratio change has been associated with a decreased risk of human obesity (Dou et al., 2022). The results obtained in this work are in accordance with the behavior of free and immobilized phenolics observed in our previous study with individual human gut microbes (Ruiz-Rico et al., 2022). Whereas the species from the phylum Firmicutes were susceptible to the phenolic compounds the growth of the species from the phyla Bacteroides and Verrucomicrobia was preserved or even enhanced in some cases.

Although the microbiota ecosystem showed different susceptibility to either free or immobilized phenolic compounds, the changes in the microbial composition generally suggest that these compounds may act as growth-stimulating factors of genera associated with beneficial effects (e.g. production of SCFAs), or could even influence competition for growth substrate and adhesion sites with microbial groups that could lead to microbial dysbiosis (Hidalgo et al., 2012).

3.3. Impact of free and immobilized phenolic compounds on defined gut microbiota ecosystem metabonomic profile

In addition to compositional analysis, metabonomic profiles derived from ^{1}H NMR analysis were obtained to determine behavioral changes of the defined microbial community after exposure to antimicrobials. Important gut microbial metabolites include SCFAs, alcohols, amines, sulfur compounds, phenols, and indoles (Oliphant & Allen-Vercoe, 2019). The overall metabonome was significantly affected by all antimicrobials and influenced by their mode of administration, according to the PCA results (Fig. 5). Treatment with the two concentration levels of the phenolic compounds resulted in different metabolic responses for the samples treated with the free or immobilized compounds, as indicated by the bimodal clustering of samples: a group with the samples treated with free antimicrobials and a second group containing the rest of the samples (control samples and samples treated with immobilized phenolic compounds). This separation was more pronounced for the high concentration (2 mg/mL) of phenolic compounds than for the low concentration (0.5 mg/mL).

The metabolite concentrations that were significantly different between the forms of administration of the different phenolic compounds are shown in Fig. 6 and Figs. S3 and S4, Supplementary Information. Eugenol treatment (Fig. 6) resulted in significant concentration changes for several metabolites derived from the metabolism of macronutrients present in the media and related to the metabolism of the phenolic compound. Exposure to free eugenol resulted in an increase in sugars and amino acids (Fig. 6A and B) in comparison to control samples, indicating a reduction of fermentation involving these substrates, potentially due to the partial inhibition of the microbial population of the ecosystem. As well, free eugenol resulted in a decrease of fermentation by-products (Fig. 6C and D) such as SCFAs (acetate and valerate) and carboxylic acids (3-phenylpropionate, 5-aminopentanoate and desaminotyrosine), with concomitant increases in the fermentation by-products formate and succinate (data not shown). The concentration of other relevant SCFAs, including butyrate and propionate, were consistent regardless of treatment (data not shown). Lipid metabolism (Fig. 6E) was also affected by free eugenol, with an increase of choline (a derivative of phospholipid breakdown) and a decrease of cholate (a primary bile acid). Ethanol and methanol concentrations increased after exposure to free eugenol, and this effect could be related to a decrease of gut microbiota species responsible for the alcohols detoxification (Fig. 6F). Apart from the metabolites derived from fermentation of nutrients, evidence of the biotransformation of eugenol was also seen in the metabolome profile (Fig. 6G). High concentrations of homovanillate (0.42 ± 0.02 and 2.49 ± 0.20 mM for the 0.5 and 2 mg/mL concentrations, respectively) and, to a lower extent, vanillylmandelic acid were found in the samples exposed to free eugenol. These metabolites could have been the result of decarboxylation reactions carried out by gut microbiota to produce phenylacetic acids using eugenol as substrate (Catalkaya et al., 2020; Selma, Espín, & Tomás-Barberán, 2009).

On the other hand, the metabolome of the tested ecosystem was only slightly influenced by exposure to immobilized eugenol. A decrease in fermentation by-products, including carboxylic acids (3-phenylpropionate and desaminotyrosine) and ethanol, was found for the samples treated with the immobilized forms of eugenol (Fig. 6D and F). As well, the concentration of the bile acids, cholic acid and glycocholic acid, increased (Fig. 6E). This rise could be related to the increase of the abundance of members of the *Bacteroides* genus which have the capacity, among other commensal bacteria, to deconjugate primary bile acids (such as cholic acid) and metabolize them into secondary bile acids (such as glycocholic acid) (Hylemon, Harris, & Ridlon, 2018; Jandhyala et al., 2015). Apart from the fermentation by-products, the biotransformation of eugenol (Fig. 6G) was different in the samples exposed to immobilized compounds. The covalent immobilization of eugenol on the carriers avoids its release in the media and, therefore, hinders its biotransformation by gut microbiota. In fact, only a concentration of 0.02 ± 0.01 mM of homovanillic acid was detected in the samples treated with immobilized eugenol while vanillylmandelic acid was not detected at all.

Vanillin treatment (Fig. S3, Supplementary Information) produced a lower impact on the metabolome profile of the defined community than eugenol. The metabolism of macronutrients present in the media mainly changed in terms of lipid metabolism, with a reduction of choline and an increase of the concentrations of bile acids in the samples treated with immobilized vanillin. With respect to the metabolism of the phenolic compound, different metabolites derived from vanillin biotransformation can be found in the metabolome profiles of free vanillin group, including vanillic acid (0.80 ± 0.11 mM), homovanillic acid (0.07 ± 0.0 mM), vanillylmandelic acid (3.23 ± 0.12 mM), 4-methylcatechol (1.56 ± 0.06 mM), and pyrocatechol (0.93 ± 0.17 mM). All of these derivatives were found in the samples treated with free vanillin, while only 4-methylcatechol (from 0.0 ± 0.0 to 0.11 ± 0.01 mM) and homovanillic acid (0.01 ± 0.0 mM) were detected in the samples treated with immobilized vanillin in low concentration.

Significant changes in the concentration of metabolites after treatment with ferulic acid (Fig. S4, Supplementary Information) were seen

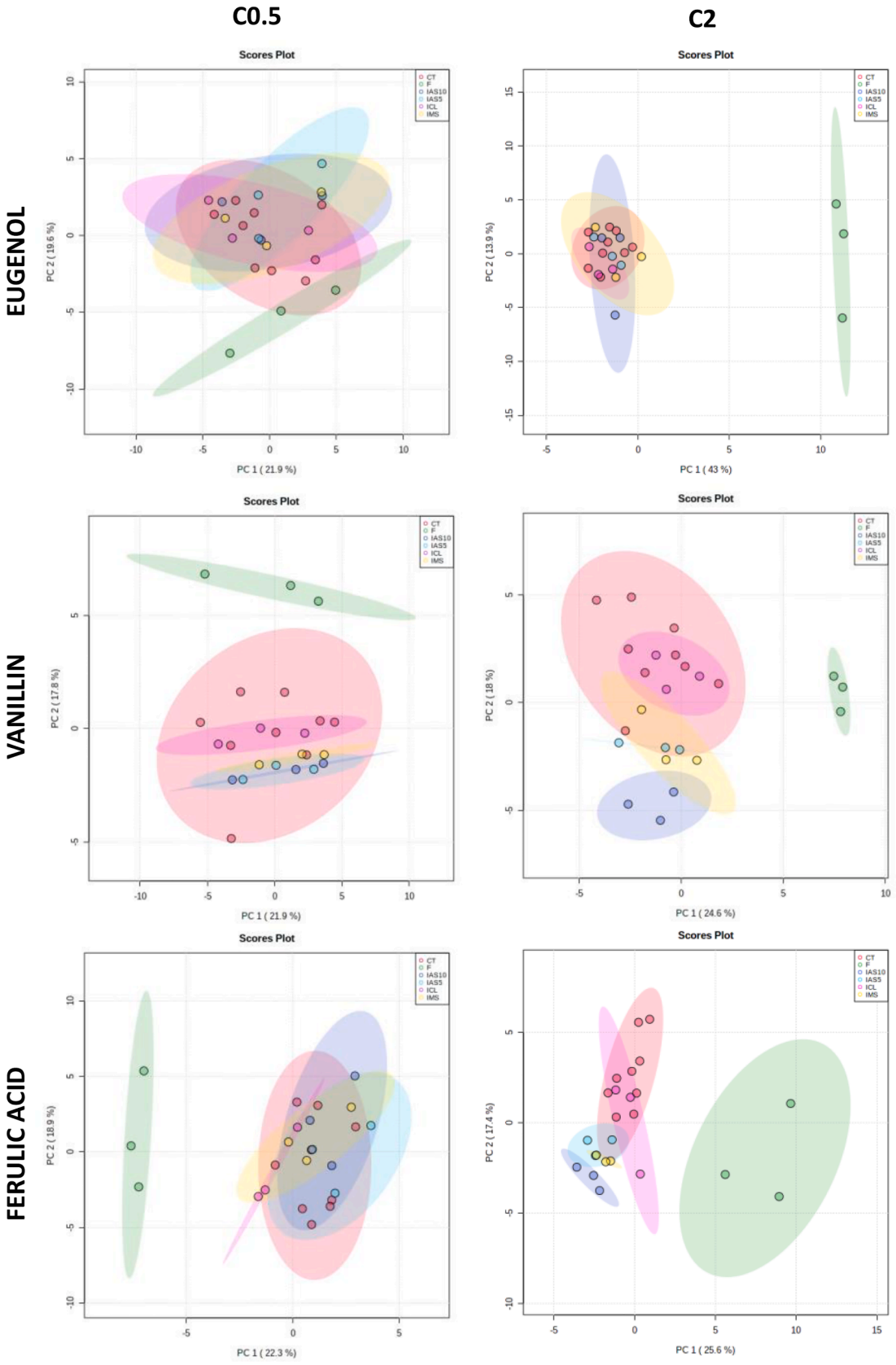

Fig. 5. Principal Component Analysis (PCA) of metabonomic data after exposure to 0.5 and 2 mg/mL of eugenol, vanillin and ferulic acid. Coloring is used to distinguish the experimental groups, as indicated. CT: control samples, F: samples treated with the free antimicrobial, IAS10: samples treated with the antimicrobial immobilized on 10 μm-amorphous silica, IAS5: samples treated with the antimicrobial immobilized on 5 μm-amorphous silica, ICL: samples treated with the antimicrobial immobilized on cellulose, IMS: samples treated with the antimicrobial immobilized on mesoporous silica.

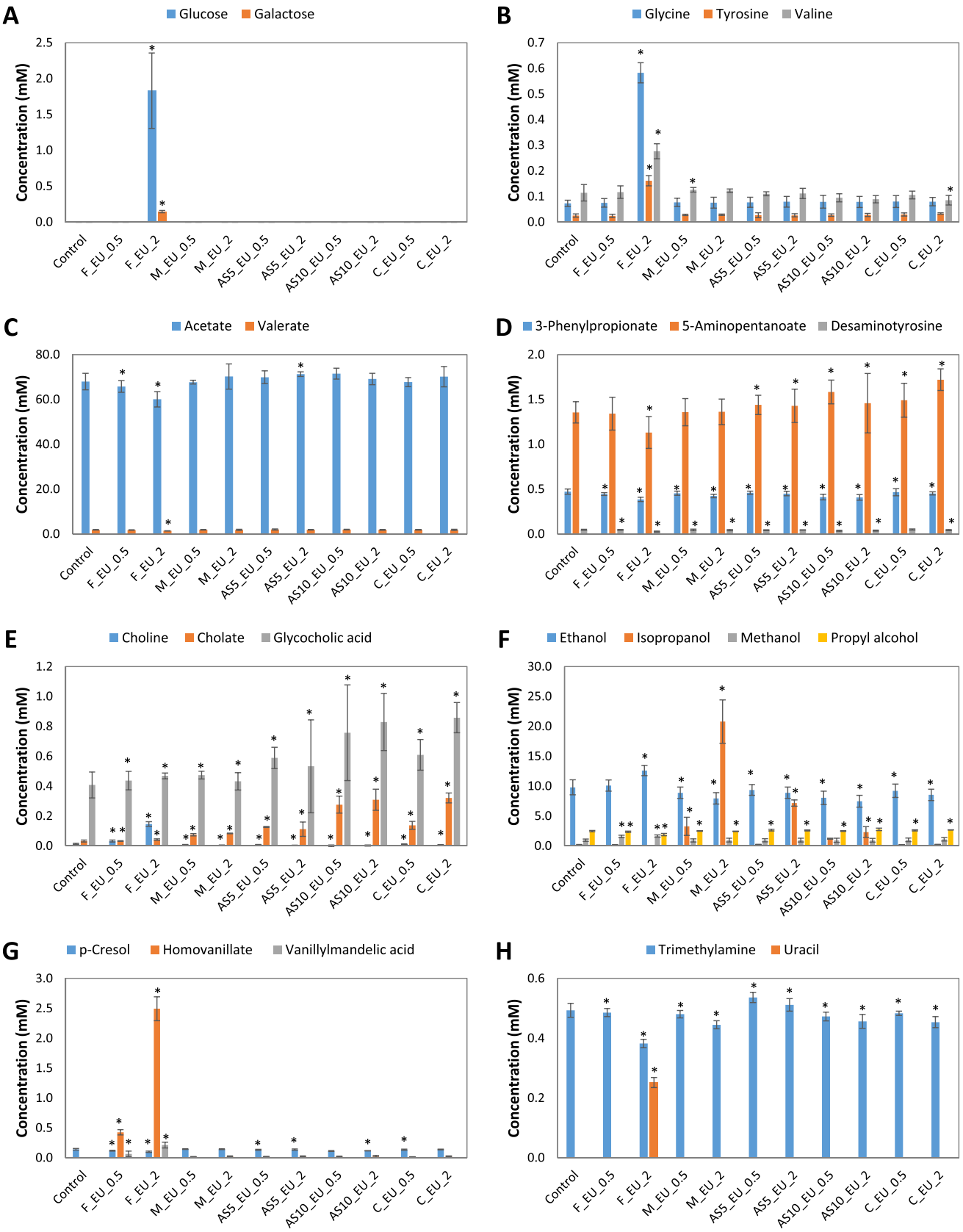

Fig. 6. Concentration (mM) of metabolites significantly affected by exposure to 0.5 mg/mL or 2 mg/mL of eugenol (EU) in its free form (F) or immobilized in mesoporous silica particles (M), 5 μm-amorphous silica particles (AS5), 10 μm-amorphous silica particles (AS10) and cellulose particles (C), in comparison to control samples. Metabolites are grouped by category: (A) sugars, (B) amino acids, (C) SCFAs, (D) carboxylic acids, (E) lipid-related metabolites, (F) alcohols, (G) phenolic compounds, and (H) others. The mean of three replicate experiments are shown, with error bars showing the standard deviation observed. (*) $p < 0.05$ indicates significant differences compared to the control.

in fermentation by-products such as formate, isopropanol, methanol, desaminotyrosine and methylamine. Free ferulic acid produced an increase in concentrations of alcohols and methylamine. Similarly to eugenol and vanillin samples, an increase in the concentration of bile acids was produced by treatment with immobilized ferulic acid. Biotransformation of ferulic acid resulted in the detection of homovanillic acid (6.42 ± 0.53 mM), vanillylmandelic acid (0.37 ± 0.07 mM), 3,4-dihydroxybenzeneacetate (1.60 ± 0.08 mM) and 4-methylcatechol (0.06 ± 0.03 mM) on the free ferulic acid group. Again, only homovanillic acid (0.02 ± 0.0 mM) was detected in samples treated with the ferulic-functionalized supports in very low concentration. According to the literature, hydroxycinnamic acid conversions by gut microbiota can be observed by reactions such as β-oxidation, decarboxylation and dehydroxylation. Reported ferulic-metabolites produced by gut microbes include vanillin, vanillic acid and 2-(3-hydroxyphenyl)acetic acid, among others (Ozdal et al., 2016; Selma et al., 2009).

It should be noted that monomeric phenolic compounds, such as those used in this study, can be absorbed through the gastrointestinal barrier by passive diffusion, enter the enterohepatic circulation, be transformed in the liver and return to the intestine (Ozdal et al., 2016). Hence, the concentration of these molecules in the gut in a real environment could be diminished and, therefore, changes in the composition and function of the microbiota may differ. Unlike free phenolics, the impact of immobilized phenolic compounds should be more consistent because the covalent grafting to carriers prevents their release; this is one of the most important advantages of the developed antimicrobial systems based on covalent immobilization (Ruiz-Rico et al., 2017). Thus, the absorption and biotransformation of the antimicrobial compounds are hampered, as is demonstrated in this work by the very low concentrations of phenolic-derivatives detected in the samples treated with the immobilized phenolic compounds.

Lastly, the presence of uracil in the samples treated with 2 mg/mL of the free phenolic compounds could favor the growth of some pathobionts (i.e. resident autochthonous bacterial species having conditionally pathogenic characteristics) capable of using uracil that may, in turn, induce inflammation (Lee et al., 2013). In contrast, the decrease of the concentration of p-cresol, a phenolic metabolite that has been associated with the decrease of the integrity of the gut epithelium (Oliphant & Allen-Vercoe, 2019), after the exposure to the different forms of the phenolic compounds could be beneficial for host health.

4. Conclusions

Evaluation of the impact of free and immobilized phenolics on a defined gut microbiota community indicated their influence on the composition and metabonomic profile. The two modes of presentation produced different impact on the ecosystem, but both resulted in an enhancement of the abundance of genera associated with beneficial effects. The metabonome profile was also significantly affected by exposure to phenolics with changes in metabolites derived macronutrients' metabolism and related to the biotransformation of the phenolic compound. Despite the impact of phenolic compounds described, the effect of free phenolics in the gut would be limited in a real scenario because they can be easily absorbed in the small intestine. Another limitation is that the concentration used in this study may not reflect the concentrations in a food product as a result of their strong influence on the sensory food properties that limit their dose. In contrast, the immobilization of phenolic compounds on carriers prevents their release and is expected to avoid these limitations. Nevertheless, future studies will focus on *in vitro* digestion of the free and immobilized compounds before evaluating their impact on the gut microbiota to overcome the limitations of the current study.

Finally, the use of a defined gut community as an *in vitro* model to evaluate the biocompatibility of food additives is a robust approach that allows a detailed study of microbial dynamics. Given the essential role of the human microbiota in health, the conclusions of this work reflect the need to study new compounds or materials that could be ingested with food by consumers and their potential influence on human health, through the use of toxicity studies employing *in vitro* models of human cells and gut microbiota, and adjunct to existing *in vivo* studies.

CRediT authorship contribution statement

María Ruiz-Rico: Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Simone Renwick:** Methodology, Data curation, Writing – review & editing. **Sarah J. Vancuren:** Methodology, Data curation, Writing – review & editing. **Avery V. Robinson:** Resources, Methodology, Writing – review & editing. **Connor Gianetto-Hill:** Methodology, Writing – review & editing. **Emma Allen-Vercoe:** Methodology, Resources, Supervision, Writing – review & editing. **José M. Barat:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The author declare the following financial interests/personal relationships which may be considered as potential competing interests: Emma Allen-Vercoe 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.

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134363.

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