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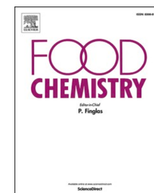

Bridging the gap of gut microbiome effects of new food additives in food risk assessment: MICODE gut model to test new chitosan from seafood waste

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ABSTRACT

EFSA and FAO outlined guidelines to address data and methodological gaps in assessing the impact of food compounds on gut microbiota (GM) and human health, emphasizing the need for *in vitro* GM models and standardized biomarkers to integrate GM data into food safety risk assessments. Food texturizers are widespread in processed foods enhancing texture and stability, yet their effects on GM remain unclear. This study investigates three additives: a chitosan from seafood waste (CHIW), a chitosan standard (CHIC), and hydroxypropyl methylcellulose (HPMC), using fructo-oligosaccharides (FOS) as a prebiotic control. A standardized *in vitro* gastrointestinal model simulating digestion and colonic fermentation, combined with qPCR and GC–MS, assessed their influence on microbial composition and metabolic output, under the hypothesis that distinct carbohydrate polymers differently affect GM. CHIC and CHIW, derived from crustacean waste, promoted beneficial bacteria (*Clostridium* group IV, *Bifidobacteriaceae*) and boosted SCFAs production, including butyrate and propionate, with CHIW showing stronger prebiotic effects. HPMC was associated with reduced abundance *of Lactobacillaceae* and harmful metabolites. These findings support our hypothesis, demonstrate the responsiveness of *in vitro* GM models to dietary polymers, and reinforce the potential of GM-based screening in food safety assessment, suggesting native chitosan as a promising alternative to synthetic additives.

1. Introduction

Food texturizers are substances added to food products to modify and improve their texture, stability, and mouthfeel. Commonly used in processed foods, they include hydrocolloids like gums, starches, and cellulose derivatives, which help maintain consistency, prevent separation, and enhance the overall sensory experience.

Although, the large employment in the food industry and the recognition as GRAS (generally recognized as safe) food products and their daily intake for consumers, the effect on human health, in particular intestinal well-being and in particular the gut microbiota (GM) is largely unknown, but is ramping concerns due to recent consolidation of the correlations among certain emulsifiers and texturizers and obesity (Salame et al., 2024), as well as food dyes and risk of cancer (Srour et al., 2023).

Chitosan, a deacetylated derivative of chitin predominantly sourced from crustacean exoskeletons, has garnered significant interest in the food industry for its potential applications as a fat-binding agent, aiding in weight management. Beyond its physicochemical properties, chitosan exhibits notable antimicrobial activities, influencing the composition of the GM. Studies have demonstrated that chitin-glucan can modulate the abundance of beneficial bacteria such as lactobacilli and bifidobacteria, while inhibiting the growth of some opportunistic bacteria, such as *Enterobacteriaceae*. This modulation leads to an increase in the production of beneficial metabolites, including acetic and butyric acids, which are essential for maintaining intestinal health (Marzorati et al., 2017).

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Considering that every year around 6–8 million tons of waste is generated as a consequence of crustaceans processing, the extraction of chitosan is surely an interesting strategy for increasing the sustainability of the food sector. However, the traditional chemical extraction process of chitosan involves various steps, such as demineralization, deproteinization and deacetylation, that require strong acids and bases and high temperatures. Depending on the source, the season, extraction process and possible modification strategies, physico-chemical and structural properties of chitosan such as deacetylation degree, molecular weight, solubility, particle size might be very different, altering its biological and functional effect (Pinheiro De Aguiar et al., 2021). It has also been observed that chitosan's modulating capacity on GM is promoted differently depending on the weight and degree of deacetylation of the molecule (Guan & Feng, 2022; Zheng & Zhu, 2003).

Hydroxypropyl methylcellulose (HPMC) (E464) is an additional example of food additives that influence GM composition. HPMC, a cellulose derivative, is widely used as a thickening and emulsifying or texturizing agent in various food products and is not absorbed by the small intestine, reaching intact the colon. Although HPMC is considered safe by EFSA (Bampidis et al., 2020), research has shown that HPMC can modulate GM with its laxative action in a different extent depending on specific gut ecological status, generating also side effects (Cox et al., 2013; Ursino et al., 2011). It is also reported that synthetic emulsifiers and texturizers, including carboxymethylcellulose (CMC), can reduce beneficial bacteria and compromise intestinal barrier integrity, potentially leading to conditions such as inflammatory bowel diseases (Bevilacqua et al., 2024).

Recognizing the complex interactions between these additives and GM is essential for assessing their safety and efficacy as food ingredients. While some additives offer health benefits by promoting the growth of beneficial bacteria and the production of health-promoting metabolites, others may adversely affect gut health by disrupting the microbial balance. This underscores the need for comprehensive research to elucidate the effects of various food additives on GM composition and function. Such knowledge is crucial for informing regulatory guidelines and aiding consumers in making informed dietary choices (Seto et al., 2025).

There is considerable evidence showing that dietary components (xenobiotics), including food additives and contaminants, adversely affect GM, potentially leading to metabolic or inflammatory disorders. However, substantial data and methodology gaps hinder the development of RA (Risk Assessment) approaches to evaluate the impact of food-related compounds on GM and their subsequent effects on human health. In a recent EFSA external opinion, Moreno-Arribas et al. (2024) proposed a roadmap for action to i) strengthen the nascent evidence relating to effects on/by gut microbiomes; and ii) list identified key knowledge gaps and provide guidance on how to experimentally approach these urgent research needs.

Another EFSA editorial, by Bronzwaer et al. (2025), addressed the data and methodological shortcomings that limit health impact risk assessment. The authors suggested combining current developments in microbiome science, such as *in silico* and *in vitro* cell systems, as well as omics, into research and innovation, because these methods can improve food risk assessments by including gut microbiome data into food safety dossiers.

Other important features to highlight are: i) the importance of generating robust datasets form omics technologies, because there is a lack of reproducible and comparable data obtained using standardized methods and technologies; ii) the importance of identifying and define biomarkers to measure relevant GM changes or GM-related host alteration, because is an essential need for using GM data in risk assessment (FAO, 2024). Among, intestinal model, the use of static batch fermentation models can provide a fast screening of the GM, assuring a basis for high throughput (FAO, 2024).

The aim of the present study is to extend the knowledge on how food texturizers influence the balance and well-being of the intestinal ecosystem, with a particular focus on assessing potential risks to the human GM. To achieve this, we combined personalized *in vitro* fermentation models with high-resolution omics analyses to obtain a detailed, rapid and reproducible tool to improve RA. As a case study, we investigated a prototype of chitosan recently developed from seafood waste of the Adriatic Sea, in comparison to other food additives. qPCR values of different microbial taxa and metabolomic data generated through the MICODE (multi-unit *in vitro* colon model) platform were integrated to evaluate microbial shifts and metabolic outputs, providing insights into how these additives may modulate GM functions and in order to assess their food safety toward the human GM. The GM of human colon was obtained pooling fecal donations of healthy volunteers. This approach aims to support the development of safer food texturizers that contribute to healthier dietary solutions, using cutting-edge biotechnology as the reliable MICODE platform and reducing animal testing in food science.

2. Materials and methods

2.1. Experimental samples

Chitosan commercial (CHIC) samples were purchased from a commercial supplier (Merck, Darmstadt, DE). Hydroxypropylmethylcellulose (HPMC) was purchased from a commercial supplier (BioLine srl, Rovigo, IT); Frcuctooligosaccharides (FOS) from chicory was purchased from a commercial supplier (Merck, DE).

All samples were analytical grade, except chitosan from seafood waste (CHIW). CHIW was recovered from mantis shrimp (*Squilla mantis* L.) by-products using a thermochemical method which included demineralization and deproteinization process as described by Tolaimate et al. (2003), followed by deacetylation process according to the procedure of Rao et al. (2007). HPMC is a semi-synthetic polymer derived from cellulose. HPMC Cellulose is chemically modified to produce HPMC. Hydroxyl groups on the cellulose are replaced with methoxy (CH3O—) and hydroxypropyl (CH2CH(*OH*)CH3) groups with a degree of susbstitution (DS) of 0,2 a 1,5 of carboxymethylic groups (—CH2COOH) for unit of anhydroglucose.

2.2. Characterization of chitosan samples

Since the physicochemical properties are decisive for the functional and biological performance of chitosan, a comparative analysis of the key parameters for the two samples (CHIC and CHIW) was carried out. The degree of deacetylation (DD%) of chitosan was determined by FTIR spectroscopy according to the method proposed by Brugnerotto et al. (2001). The viscosity-average molecular weight (Mv) of chitosan was calculated from the intrinsic viscosity [η] measured by a single-point method using the Mark-Houwink-Sakurada equation. (Baris et al., 2023). The colorimetric analysis was performed using a spectrophotocolorimeter and the whiteness index (WI%) was calculated according to the equation of Rhim et al. (1999) based on the CIELAB colour space coordinates. Ash content and solubility were evaluated following the procedures described by Kumari et al. (2017). Particle size distribution was measured using a calibrated standard sieve.

2.3. Experimental pipeline

In this work, we employed an *in vitro* intestinal system coupling simulation of human oro-gastric-duodenal digestion, Infogest® protocol (Minekus et al., 2014), and colonic fermentation, MICODE protocol (Gianotti et al., 2025; Nissen et al., 2021). Data were obtained by qPCR enumeration and metabolomics (gas-chromatography/mass-spectrophotometry) and analyzed and combined with multivariate statistical analysis (Nissen et al., 2020, 2024) in R and ShinyR environment.

A flowchart outlining the research procedure is shown in Fig. 1. Specifically, both experimental samples and controls underwent for two independent times oro-gastro-duodenal digestion and colonic

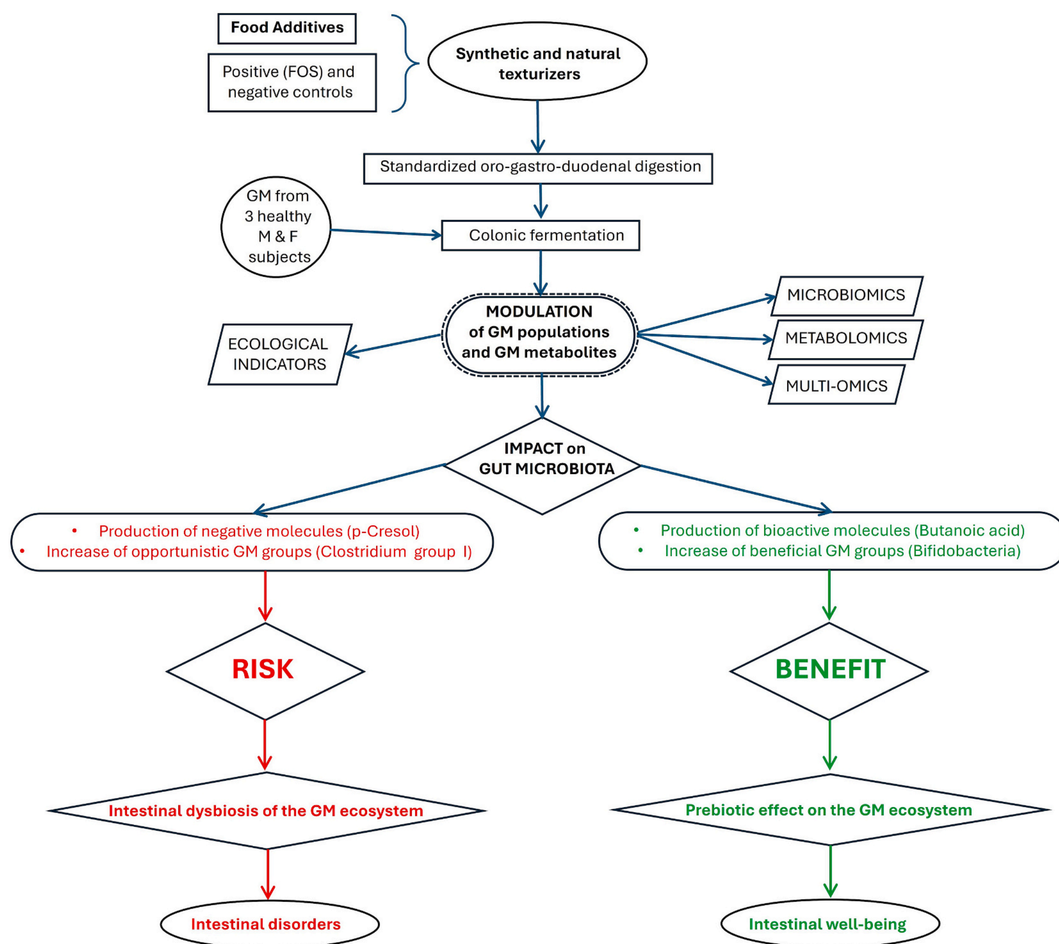

Fig. 1. Flow chart of the work.

fermentation with human colonic microbiota Each colonic fermentation was conducted with GM obtained from fresh fecal donations of three different healthy volunteers. The full procedure was repeated for two independent periods, using GM from six different donors in total. in a controlled *in vitro* setup. Colonic fermentation effluents were collected at the start, at an intermediate stage, and at the conclusion of fermentation, and were analyzed to assess the populations and abundances of the colonic microbiota, as well as the production of microbial metabolites, including the total volatilome. Multivariate analysis was conducted to facilitate comprehensive comparisons across the samples and time points.

2.4. Human gut microbiota recovery and preparation

Human GM was sourced from fecal samples of healthy adult individuals. The sample collection followed established protocols from previous studies (Connolly et al., 2012; Gianotti et al., 2025; Marangelo et al., 2025; Nissen et al., 2023). Specifically, samples were collected on two occasions, each time from three different participants (six in total), aged between 30 and 5 years, who had not received antibiotic treatment for at least 3 months and had not deliberately consumed prebiotics or probiotics prior to the experiment. To conduct the experiment, equal amounts of fecal material from the three donors were pooled to create a sample, rather than analyzing each donor individually. The participants were fully informed about the purpose and procedures of the study and verbally consented to the use of their fecal samples, in line with the Bioethical Committee of the University of Bologna. Fecal samples (Bristol stool types 2–5) were provided during the experiment. For sample collection, donors were supplied with a collection kit, which included a stool collector (Sarstedt AG & Co. KG, Nümbrecht, Germany) and an anaerobic jar containing an O_2-consuming catalyst (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA). The samples were promptly transported to the laboratory and processed within 2 h. A 10% (w/v) GM suspension was prepared immediately before the experiment by homogenizing 2 g of each donation (6 g in total) in 54 mL of pre-reduced phosphate-buffered saline (PBS) (Cattivelli et al., 2023; Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020), followed by two washes of the pellet in PBS (16,000×g for 5 min) (Gianotti et al., 2025). The study was accomplished adhering to bioethics methods required at University of Bologna and approved by the Bioethical Committee of the University of Bologna (protocol code Prot. n. 0061183 and approved on 15 March 2021).

2.5. In vitro gut model with gut microbiota of healthy adults for food texturizers assessment

Food texturizers and control samples were subjected to digestion following the INFOGEST protocol (Minekus et al., 2014). A total of 5 g from each food texturizer sample was processed for 245 min (2 min of oral digestion, 120 min of gastric digestion, and 120 min of intestinal digestion) at 37 °C. Enzymatic treatments included simulated saliva (75 U/mL α-amylase), simulated gastric juice (2000 U/mL pepsin), and simulated pancreatic juice (10 mM bile, 100 U/mL pancreatin) at respective pH levels. Easch sample was digested three times, generating a triplicate ($n = 3$).

After digestion, the triplicate of a food samples digestate was pooled and centrifuged (16,000×*g*, 7 min) and 1 mL of the precipitate emulsion (representing the denser part of the digestate not absorbed by the small

intestine and that reaches the colon) was recovered for (human colon microbiota fermentation using the MICODE protocol and MICODE apparatus (Nissen et al., 2021). The MICODE protocol simulates short-term (24 h) batch fermentation in remotely controlled single bio-reactors under precise controlled and monitored conditions reflecting the human proximal colon (Nissen et al., 2023). Each tested digestate sample was fermented independently in a bioreactor of MICODE. The experiment was replicated two times, with GM from three different groups of donors.

Experiments were conducted in accordance with published procedures (Connolly et al., 2012; Gianotti et al., 2025; Marangelo et al., 2025; Nissen et al., 2024; Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020), which included the preparation of the basal medium, preparation of the inoculum from pooled fecal samples, maintaining of *in vivo* physiological conditions. These essential conditions are necessary to mimic the proximal colon niche, namely; i) anaerobiosis, measured with dissolved oxygen (DO_2) at 0.0–0.1% w/v and provided by filtered-nitrogen flushing directly at the bottom of the vessel and inside the 100 mL of volume; ii temperature at 37 °C, controlled with a Peltier thermostat regulating temperature at interval of 0.1 °C (Gettinge, Sweden); iii) the pH adjusted to 5.75 and maintained throughout the experiment with peristalsis pumps providing 0.32 μm filtered 0.5 M NAOH or 0.5 M HCl solutions. Once simulated physiological conditions were stabilized, 10 mL of the MICODE working solution (containing 1 mL of digestates of food texturizers suspended in 9 mL of fecal slurry washed two times by centrifugation at 6000*g* per 5 min in O_2-reduced PBS) was aseptically injected into each bioreactor. MICODE, thanks to precise Applisense sensors (Getinge SA, Goteborg, Sweden) is able to record every ten second values of temperature, pH and DO_2, monitor and adjust them to maintain strict settings. The digestates used included CHIC, CHIW, HPMC, FOS, and BC (no substrate). pH changes were monitored using pH sensors and recorded with Lucullus 3.1 software (PIMS, Gettinge BV, NL). An adaptation of the GM from *ex vivo* to *in vitro* condition was considered and was defined as the beginning of colonic fermentation, namely the baseline (BL) (Connolly et al., 2012), that for these experiments was determined after 1.46 ± 0.13 h and fermentation was allowed to proceed for 25.46 h, with sampling at BL, an intermediate time point (T1 = 16 h), and the endpoint (EP). The basal nutrient medium (per liter) contains two grams of peptone and two grams of yeast extract as primary nitrogen and growth factor sources, 0.1 g of sodium chloride (NaCl), 0.04 g of dipotassium hydrogen phosphate (K_2HPO_4), 0.04 g of potassium dihydrogen phosphate (KH_2PO_4), 0.01 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.01 g of calcium chloride hexahydrate ($CaCl_2 \cdot 6H_2O$), two grams of sodium bicarbonate ($NaHCO_3$), two milliliters of Tween-80, 0.05 g of hemin (which is first dissolved in one milliliter of 4 M NaOH), ten milliliters of vitamin K solution, 0.5 g of L-cysteine hydrochloride, and 0.5 g of bile salts (a mixture of sodium glycocholate and sodium taurocholate). The medium is adjusted to pH 7.0 prior to autoclaving, and once cooled, two milliliters of a 0.025% (w/v) resazurin solution are added for redox indication (Connolly et al., 2012; Gianotti et al., 2025).

Sampling was carried out using a double-syringe system to avoid disturbing the ecosystem, and experiments were monitored continuously. The samples were centrifuged, and the supernatant was stored for SPME-GC–MS analysis, while the pellets were washed in PBS and used for microbial DNA extraction. DNA extraction was performed immediately to preserve the *Bacillota* content. All samples were stored at −80 °C prior to analysis. For qPCR analysis each biological duplicate of a sample was tested three times ($n = 6$), while for GCMS each biological duplicate of a sample was tested two times ($n = 4$). Quality controls of the MICODE model were also considered, as previously described (Gianotti et al., 2025).

2.6. DNA and gut microbiota analyses

DNA was extracted from the MICODE effluates at each time points (BL, T1, and EP) using the Nucleo Spin DNA stool Kit (Macherey Nagel, Darmstadt, Germany). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK) and samples of high purity were chosen. DNA samples were extracted at the baseline time point, intermediate time point and endpoint of GM clonic fermentation and 10 ng of high quality DNA of each sample were used for quantitative PCR (qPCR) analyses.

To quantify, by means of cells/mL of bioreactor volume, the changes in the GM bacterial populations after colonic fermentation, enumeration of bacterial groups was made by qPCR and SYBR Green I chemistry, following previous protocols (Gianotti et al., 2025; Tamargo et al., 2022; Tanner et al., 2014; Westfall et al., 2018). Specifically, some bacterial groups were selected as generally accepted indicators of eubiosis or dysbiosis of GM, and their perturbations were considered closely correlated (directly or inversely) to the prebiotic potential of foods (Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020). 13 different bacterial taxa, (Table S1), were assessed by qPCR on a QuantStudio5® System with SYBR Green I chemistry (Applied Biosystem, Thermo Fisher, USA). Gene standards were prepared from the relative PCR run amplicons of the target bacterial species using the GeneJet amplicon purification kit (Thermo Fischer Scientific, USA) as previously described (Modesto et al., 2011; Nissen et al., 2024). qPCR reactions were set up as previously published (Cattivelli et al., 2023). Results were expressed as mean values of sextuplicate, as obtained by two biological replicates and three technical replicates.

2.7. Volatilome analysis

The analysis of volatile organic compounds (VOCs) was conducted using an Intuvo 9000 GC System (Agilent Technologies, Santa Clara, CA, USA) coupled with an 5977B GC/MSD System mass spectrometer operating in electron impact mode (ionization voltage of 70 eV) (Agilent Technologies, USA). The system was equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, NL). The Solid Phase Micro-Extraction (SPME) GC–MS procedure and identification of volatile compounds were performed following previously established protocols, with minor adjustments (Nissen et al., 2023; Gianotti et al., 2025). In short, 2 mL of MICODE effluents were placed in 10-mL glass vials and supplemented with 4 μL of an internal standard mixture (2-Pentanol, 4-methyl- at 10000 ppm) to achieve a final concentration of 5 mg/L. Samples were equilibrated for 10 min at 45 °C. A carboxen-polydimethylsiloxane SPME fiber (85 μm) was then exposed to the sample for 40 min. The preconditioning, absorption, and desorption steps for SPME-GC–MS analysis, along with data processing, followed protocols outlined in previous studies (Nissen et al., 2021). The identification of compounds was carried out by matching mass spectra against available databases (NIST 11 MSMS library and NIST MS Search program 2.0, NIST, Gaithersburg, MD, USA). Each VOC was quantified relatively in percentage terms (LOD = 0.001 mg/kg) (Bonfrate et al., 2020). Additionally, the primary microbial metabolites linked to prebiotic activity in BL and EP samples were quantitatively measured in mg/kg (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2023), and results were presented as changes. All data were expressed as normalized mean values derived from duplicates in two independent experiments. Syntax of molecules name is that of the NIST 11 MS Library, where official names are reported with the first initial in capital letter, while synonyms are reported with first initial in lower case, for example: 1H-Indole, 3-methyl and skatole.

2.8. Data processing and statistical analysis

The datasets of this research were obtained from two experiments conducted in different dates and with different fresh inoculum. Each inoculum was made pooling stool donations of three different male or female volunteers. In total stool donations were obtained from six different donors. Each food additives and the blank control (BC) was

digested and fermented by pooled GM in two distinct experiments. During GM fermentation, three different time points to conduct analysis were observed, the baseline (BL), 16 h of fermentation (T1) and the endpoint (EP) at 24 h of fermentation.

The microbial enumeration results were obtained from qPCR (performances and parameters are reported in Table S2) values that were calculated as $Log_2(F/C)$ (Hill et al., 2017) applied to *in vitro* GM models (Nissen et al., 2021) and processed with MANOVA, using "time" and "food matrix" as categorical predictors, followed by a Duncan *post hoc* test ($p < 0.05$) (Table S3). The sample size of qPCR analysis was $n = 6$, as each value was computed from six replicates obtained from two biological replicas (experiments performed two times in different period and with different fresh inoculum) and three technical replicas (three wells of qPCR plates). The metabolomic results were obtained from GCMS values that were calculated by comparison with a known quantity of chemical standard and processed with MANOVA, using "time" and "food matrix" as categorical predictors, followed by a *post hoc* Tukey HSD test ($p < 0.05$). The sample size of GCMS analysis was $n = 4$, as each value was computed from four replicates ($n = 4$) obtained from two biological replicas and two technical replicas (two vials and two resulting chromatograms for SPME-GCMS).

Statistical analysis was conducted for all the samples, time points and tested conditions, comparing samples to controls and beginning of GM fermentation time. The multi-omic results were obtained by non-parametric Spearman's rank correlation analysis using single sample dataset with significant ANOVA variables. The results were visualized as a heatmap with cluster dendrograms Spearman's rank Correlations among bacteria and molecules (Fig. 4) were done employing the dataset of shifts and changes at T1 and EPs and preparing a two-way heatmap with no specific scale order. Spearman's rank Correlations among bacteria, molecules and technological features of chitosan samples (Fig. 5) was done employing the dataset of shift and changes of bacteria and molecules at late timepoints and the dataset of technological attributes of chitosan prior *in vitro* assessment. All datasets for ANOVA/MANOVA computation were priorly datamined for normality, using the Shapiro-Wilk's test and for homoscedasticity, using the Levene's test, as previously indicated in food science analysis (Granato et al., 2014). Detailed statistics are reported in supplemental tables. Statistical analysis and graphics were generated using Statistica v.8.0 (Tibco, Palo Alto, CA, USA), HeatmappeR (Babicki et al., 2016) and Rstudio software (Posit, Boston, MA, USA).

3. Results and discussion

3.1. Technological characterization of chitosan samples

Since the physicochemical properties are decisive for the functional and biological performance of chitosan, a comparative analysis of the key parameters for the two samples (CHIC and CHIW) was carried out. The results of charatecterisation showed that chitosan from crustacean waste (CHIW) had a higher average molecular weight (kDa), degree of deacetylation (DD%), solubility and whiteness index than CHIC, while both had a similar low ash content and fine particle size (<500 μm) (Table 1). Higher molecular weight chitosan is associated with enhanced viscosity and gel-forming properties, make it particularly suitable for food texturizing and thickening applications (Wang, Gibson, et al., 2020). In contrast, low molecular weight chitosan is often linked to higher bioactivity, particularly antimicrobial effects, due to improved mobility and interaction with microbial membranes (Pavinatto et al., 2014). However, CHIW also showed a significantly higher DD% (78.92%) and solubility (98.33%), both of which are critical parameters enhancing its bioactivity and dispersion in aqueous environments. These properties are essential for the role of chitosan as a natural preservative, as antimicrobial and antioxidant activities are positively influenced by high DD% and solubility (No et al., 2002).

Table 1
Compositions of the chitosan samples.

Analyses	Mean values ± SD* of Chitosan products	
	CHIC	CHIW
Average molecular weight (kDa)	463.14 ± 5.41^b	2108.99 ± 86.00^a
Deacetylation degree (DD%)	75.25 ± 0.63^b	78.92 ± 1.04^a
Solubility (%)	90.28 ± 0.98^b	98.33 ± 0.29^a
Ash (%)	0.69 ± 0.02^a	0.69 ± 0.03^a
Whiteness Index (WI)	79.55 ± 0.06^b	88.17 ± 0.09^a
particle size	<500um	<500um

CHIC = Chitosan from commercial supplier (Merck, Darmstadt, Germany); CHIW = Chitosan from seafood waste; *Mean values of 3 replicates and standard deviation. Different letters in the same line indicate statistically significant differences ($p < 0.05$).

3.2. Validation of MICODE protocol

To validate the MICODE experimental approach some parameters related to metabolites and microbes were monitored as quality controls (QCs). QCs for microbial taxa were relative to: i) experimental trends, plots and constant parameters recorded by Lucullus software; ii) the ability to maintain in cultivation *Clostridium* group IV and *Faecalibacterium prausnitzii*, which are extremely sensible to oxygen content (Samuel et al., 2007; Traore et al., 2019), from the BL to the EP; iii) the confirmation of the paradigm of prebiotic, in fact when the positive control FOS was assessed, GM responders were principally beneficial LAB (lactic acid bacteria) associated to production of organic acids targeted to limit *Proteobacteria* and iv) considering the volatilome, some stool ubiquitous compounds, namely thiourea, propanol, and aniline were retained at the same retention time through every chromatograms. All those indicators confirmed the validity of MICODE as *in vitro* model system to study human GM ecology exposure to food additives, such as texturizers.

3.3. Enumeration and modulation of core microbiota groups by qPCR

Quantifications of bacterial groups at the baseline are reported in Table S4, while the shift as $Log_2(F/C)$ are reported in Fig. 2. Eubacteria showed a significant change only for underrepresentation at late time points fermentation of the sample CHIW and overrepresentation fostered by HPMC and FOS (Fig. 2a). The two main bacterial phyla *Bacteroidota* and *Bacillota* showed a naturally opposite trend during colonic fermentation. In particular, the *Bacillota* phylum was significantly underrepresented just by FOS and was overrepresented by HPMC and minorly by CHIW, while was limited by CHIC. In contrast, the phylum *Bacteroidota* was significantly underrepresented by FOS and CHIC and overrepresented by CHIW and HPMC. To this extent the ratio *Bacillota/Bacteroidota*, known as F/B (former *Firmicutes/Bacteroidota*), was reduced just by FOS and CHIC. *Bacillota* and *Bacteroidota* represent 85% of total colon microbiota and the F/B ratio has been considered as an ecological indicator of eubiosis or dysbiosis depending on whether the ratio value is less or greater than 2 (Koliada et al., 2017; Vaiserman et al., 2020). Healthy subjects without dysbiosis have F/B values within the limit of 2, that means a proper balance of the two phyla. Otherwise, subjects with dysbiosis have values slightly higher than 2 and subjects with pathologies, such as IBD (intestinal bowel disease), have F/B values much greater than 2 (Magne et al., 2020) defined by excessive growth of *Bacillota*.

Our results are similar to previous findings employing pure commercial chitosan forms but are also in contrast to those revealed by native form of chitosan as is CHIW (Marzorati et al., 2017).

3.4. Quantification and modulation of eubiosis-inducing beneficial microbial groups

Considering beneficial species, we have assessed lactobacilli, bifidobacteria and *Clostridium* group IV, that are renown traditional

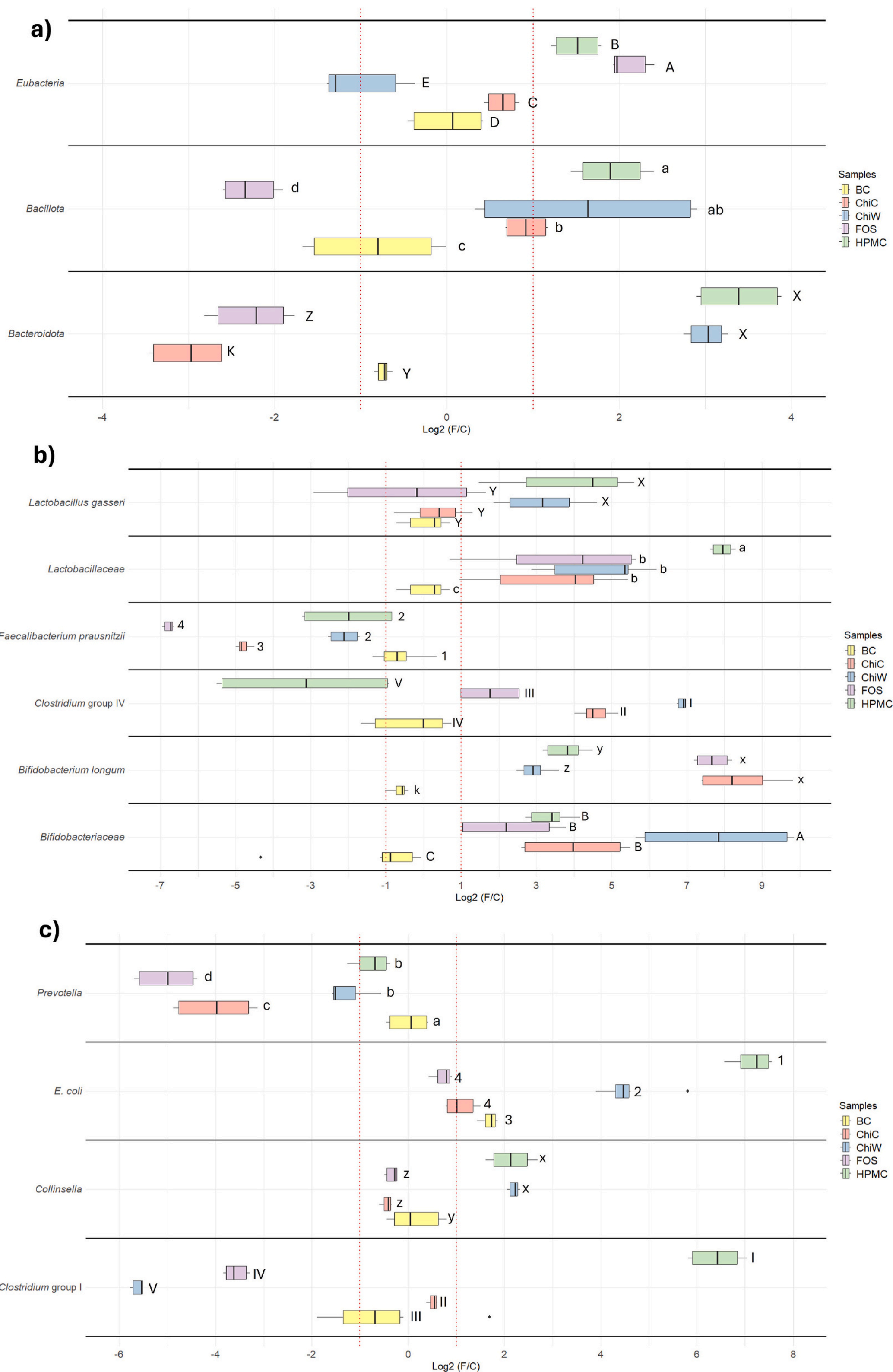

Fig. 2. Box plots of Log_2(F/C) shifts of (a) core microbiota; (b) beneficial groups and (c) opportunistic groups of the gut microbiota. Shifts are relative to quantification values recorded at the intermediate time point and at the endpoint of fermentation *versus* baseline of fermentation. Bacterial enumerations were achieved with qPCR. Boxes within the dotted red lines have no significant differences *versus* the baseline. Letters, numbers and Latin numbers indicate statistical differences of food additive samples within a single bacterial group. Sample size is $n = 6$, obtained by two independent biological replicates and three technical replicates. CHIC = Chitosan commercial; CHIW = Chitosan from seafood waste; FOS = Fructooligosaccharides; HPMC = Hydroxypropyl-methylcellulose. Baseline values in cells/mL, values of Log_2(F/C) shifts at T1 and EP and full MANOVA (Multivariate analysis of variance) with Duncan *post hoc* statistics are reported in Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

probiotics or second-generation probiotics, conferring well-being to the host (Wang, Li, et al., 2024; Wang, Wang, et al., 2024). In general, chitosan samples generated a positive GM response during colonic fermentation (Fig. 2b). This positive modulation was significant for both CHIC and CHIW with a larger range to CHIW, as both were able to significantly foster at least 5 beneficial groups out of 6 tested, namely *Lactobacillaceae, Lactobacillus gasseri, Clostridium* group IV*, Bifidobacteriaceae; Bifidobacterium longum* (p < 0.05)*,* with the exception of *F. parusnitzii,* that was significantly reduced over time (p < 0.05). The different trends of growth observed for beneficial *Clostridium* group IV and its resident *Faecalibacterium prausnitzii,* that are active butyrate producer and GM eubiosis regulators (Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020), is debated. Eventually the positive effect could be targeted to ù*Roseburia spp.* that is reported in literature to be fostered by chitin-glucans (Marzorati et al., 2017), although not analyzed in this work. HPMC, instead, was able to foster both lactobacilli and bifidobacteria groups, but not the *Clostridium* group IV. HPMC affinity was higher for lactobacilli than for bifidobacteria, and the former was stronger while the latter was weaker in comparison to the affinity of chitosan samples. These results are in line with previous findings reporting that HPMC can modulate the intestinal microbiota by reducing specific microbial populations, like *Clostridium* group IV (Cox et al., 2013).

3.5. Quantification and modulation of dysbiosis indicator opportunistic microbial groups

Colonic fermentation of the digested samples caused a reduction in the opportunistic groups of GM (Fig. 2c). CHIW exhibited a suppressive effect, significantly decreasing the relative abundance of *Prevotella*, and *Clostridium* group I (which includes *Clostridium perfringens*), all known for their association with gut dysbiosis and inflammatory conditions (Malinen et al., 2010*;* Su et al., 2022). CHIC reduced *Prevotella* more than CHIW and FOS also had the ability to decrease some opportunistic bacteria. In contrast, HPMC resulted in adverse modulation of GM, promoting the growth of 3 out of 4 opportunistic bacteria tested (*Clostridium* group I, *Escherichia coli* and *Collinsella aerofaciens*), suggesting a less favorable impact on gut health. These data support the notion that chitosan, particularly CHIW, exerts beneficial antimicrobial effects against pathobionts, which may contribute to maintaining GM eubiosis (Edo et al., 2025).

3.6. Volatilome; the complex of aromatic molecules from colonic fermentation

The volatilome of colonic fermentations of food texturizer samples (Fig. 3 and Tables S5) showed a clear distinction of the variables on a case-by-case basis for significant molecules, according to ANOVA (Fig. 3a). Additionally, with Principal Component Analysis (PCA) of single classes of VOCs it was possible to assign specific signatures and with different extent to each fermentation substrate. This means that

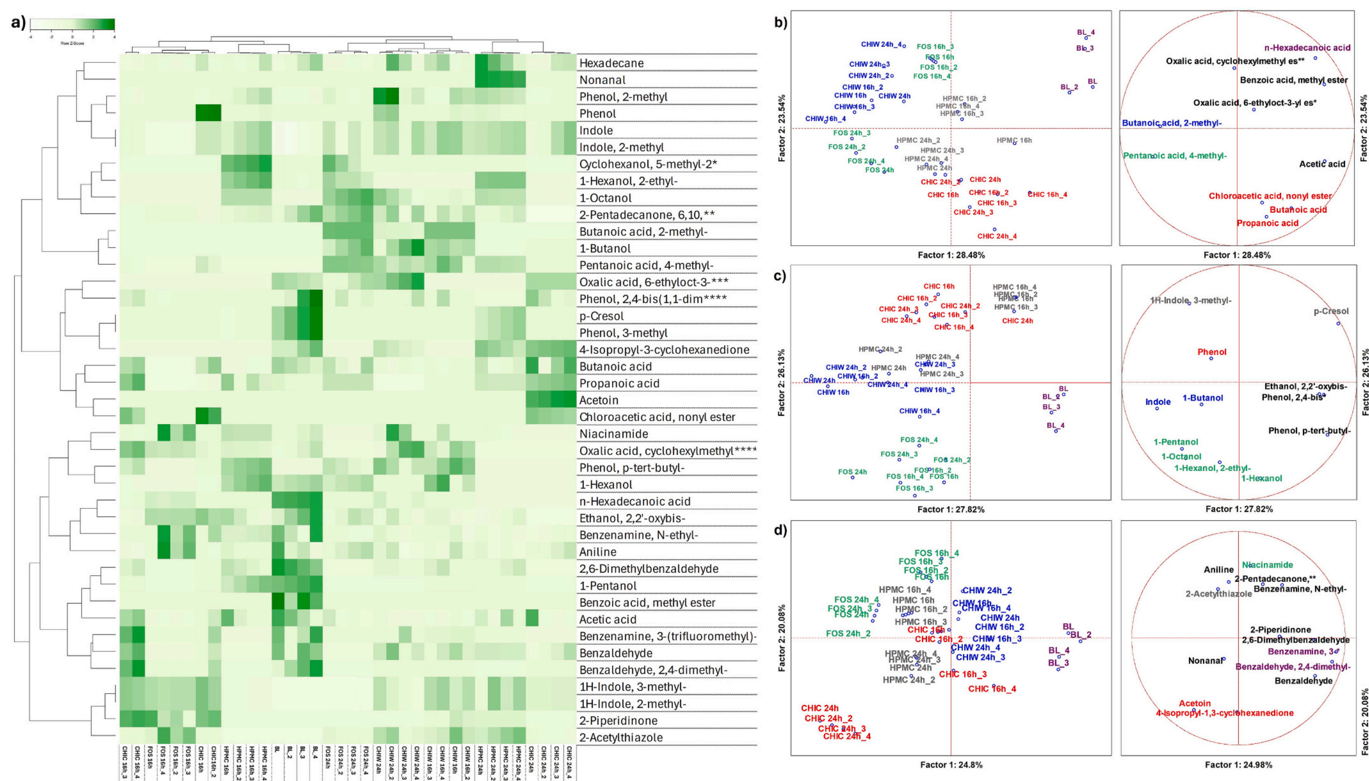

Fig. 3. Comprehensive analysis of the volatilome produced during colonic GM fermentation of digested samples. (a) Quantification heatmap of volatile organic compounds (VOCs) of samples, time points and replicates (n = 4), with Spearman rank dendrograms; (b–d) Plots of Principal component analysis (PCA) of cases on the left side and dependent variables on the right side; (b) PCA of organic acids VOCs; (c) PCA of alcohols VOCs; (d) PCA of amines, aldehydes and ketones VOCs. Colors of labels in the PCAs of dependent variables indicate cases-specific descriptors by multivariate analysis of variance (MANOVA). CHIC = Chitosan commercial; CHIW = Chitosan from seafood waste; FOS = Frcuctooligosaccharides; HPMC = Hydroxypropyl-methylcellulose; BL = Baseline values (values at the beginning of colonic GM fermentation); 16 h = 16 h of colonic GM fermentation; 24 h = 24 h of colonic GM fermentation; _2, _3, _4 = biological and technical replicates. *Cyclohexanol, 5-methyl-2 = Cyclohexanol, 5-methyl-2-(1-methylethyl)-; **2-Pentadecanone, 6,10, = 2-Pentadecanone, 6,10,14-trimethyl; ***Oxalic acid, 6-ethyloct-3- = Oxalic acid, 6-ethyloct-3-yl ethyl ester; ****Phenol, 2,4-bis(1,1-dim = Phenol, 2,4-bis(1,1-dimethylethyl); *****Oxalic acid, cyclohexylmethyl = Oxalic acid, cyclohexylmethyl tetradecyl ester. Supplemental material can be found in Table S5.

after colonic fermentation of a substrate by the human GM, different and specific VOCs with bioactive attributes are released into the colon environment and are then eventually bioavailable for the host (Dell'Olio et al., 2024). Regarding organic acid production (Fig. 3b), PCA allowed discrimination of the digested texturizer samples on: i) a time-basis, discriminating BL samples to T1s and EPs and ii) on a substrate basis, discriminating CHIW and FOS to CHIC and HPMC. Each fermentation of substrate samples had generated specific signatures of one or more VOCs, as follows; CHIW fermentation led to production of Butanoic acid, 2-methyl, FOS fermentation led to Pentanoic acid, −methyl and CHIC fermentation to Butyric and Propionic acids. Through this precise sight it is possible to predict that CHIC and CHIW after digestion and fermentation by the GM generates somehow beneficial postbiotic compounds, as butyrate and propionates which favors gut homeostasis and trigger a large variety of other health-related effects, from satiety and hungry regulation to the induction of apoptosis of damaged cells, are additionally GM energetic sources concurring to the generation of an healthy GM and intestinal well-being (Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020).

Concerning the volatilome of fermentation alcohols (Fig. 3c), which includes linear alcohols C_2-C_{10}, it can be observed that a discrimination of samples according to VOCs was defined on a time basis, as samples signatures were different before and after GM fermentation. In this PCA analysis, there are some discriminations also substrate dependent, as CHIW fermentation was described by 1-Butanol and Indole and FOS by 1-Pentanol, 1-Octanol and 1-Hexanol, thus indicating no harmful alcohols produced and in contrast to CHIC and HPMC. This former produced Phenol, while the latter produced 1H-Indole, 3-methyl (a.k.a. skatole) and p-Cresol, which are all toxic compounds for the host mucosa (Fig. 3c). Lastly, among other VOCs with bioactive attributes (Fig. 3d), it is interesting to note that FOS fermentation was described by the production of beneficial niacinamide, CHIC fermentation by unharmful acetoin and HPMC fermentation by detrimental 2-Acetylthiazole. The scenario proposed by volatilome analysis indicates that texturizer chitosan is not a risk for consumption and is better than HPMC, as chitosan products after GM fermentation generate no risky compounds in respect to HPMC. So far, the results obtained could give an help to explain the renowned risk associated with texturizers consumption, providing a mechanical insight that HPMC impacts negatively to the GM, leading to the detrimental production of phenol, skatole and p-Cresol. In particular, these compounds are generated by unfavorable GM fermentation led by *Enterobacteriaceae* and *Clostridium* group I and needs caution, because are robust biomarkers associated to dysbiosis, IBD and colorectal-cancer, as p-Cresol and skatole (Al Hinai et al., 2019; Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020; Candeliere et al., 2022; Zgarbová & Vrzal, 2023).

3.7. Production capacity of microbial metabolites with beneficial or detrimental effect toward the host

Production of main microbial metabolites of food fermentation were studied as differences in mg/kg between the baseline *versus* T1 and EP time points (Fig. 4 and Table 2). Our findings demonstrate that; first as texturizer chitosan produces an healthier impact on GM than HPMC and second, among the two chitosan samples CHIC produced an healthier impact than CHIW. Thus far, the functional strength of the tested food additives is FOS > CHIC > CHIW > HPMC. For example, HPMC

Table 2
Baseline values of quantification of VOCs in mg/kg.

VOCs ID*	Mean quantity (mg/kg ± SD)**,#	*p* value§
Acetic acid	0.50 ± 0.25	0.00383
Propionic acid	0.11 ± 0.02	0.00032
Butanoic acid	0.12 ± 0.02	0.00072
Butanoic acid, 2-methyl	<0.03 ± 0.00	0.00743
Pentanoic acid, 4-methyl	<0.03 ± 0.00	0.40767
Indole	4.42 ± 1.96	0.00561
1H-Indole, 3-methyl (a.k.a. skatole)	<0.01 ± 0.00	0.35918
Phenol	0.07 ± 0.01	0.00098
p-Cresol	6.98 ± 2.78	0.00945

*Identified by NIST 11.0 MSMS library (NIST, Gaithersburg, MD, USA); **mean quantity ($n = 4$) of microbial compounds at the baseline, *i.e.* the beginning of GM fermentation; quantified employing as chemical standard 2-Pentanol, 4-methyl; §analysis of variance with statistical significance set at $p < 0.05$. * LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg.

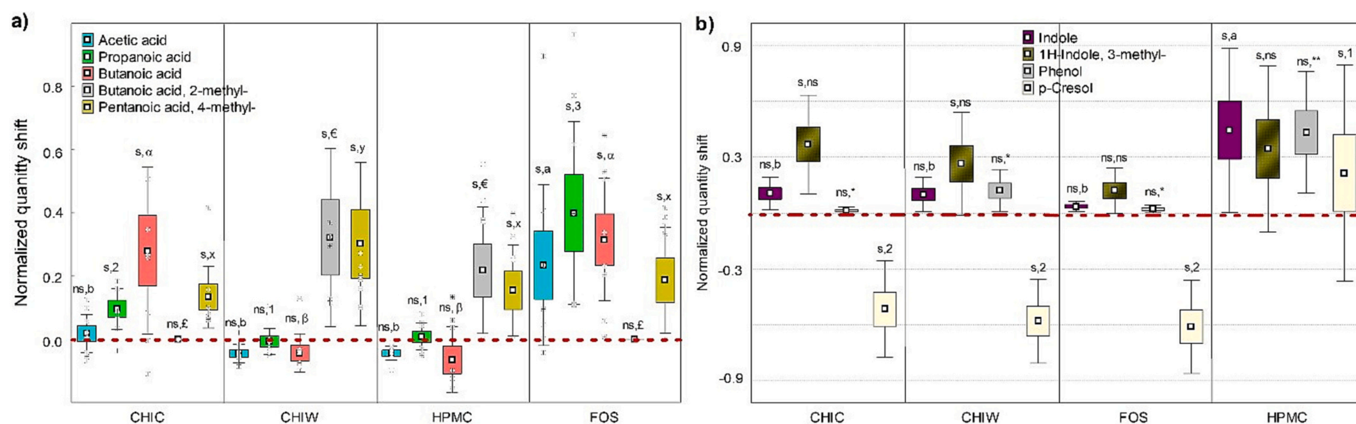

Fig. 4. Changes during colonic fermentation of a) host-positive and b) host-detrimental microbial metabolites. As determined by *post hoc* tests, above each plot, we reported a note made by two items; the first is the term 's' (significant) or 'ns' (not significant) indicating statistical significance *versus* the baseline GM fermentation (red dotted line) followed by a comma before the second item as a letter, number, or symbol indicating statistically significant differences among the various food texturizers within each compound,. Statistic was made by MANOVA and Tuckey *post hoc* test at $p < 0.05$. Plots are populated by the values of each time point and replicate, so that a plot of greater extent indicates that production of the compound occurred throughout the fermentation, while a plot of reduced extent indicates that production occurred at a specific time during fermentation. The sample size is $n = 4$, as each sample was obtained from two biological replicates and two technical replicates. Normalization of values is made for each single compound with a mean centering method, and time T1 and EP values were subtracted from BL values. To this disposition to interpret the plots results, this example can be followed: CHIC has a value of approx. 0.25 (on a scale 0.00–1.00) that means at T1 and EP time points it has produced approx. 2.5 times more the value of the Red dotted line, *i.e.* the baseline of GM fermentation, thus CHIC impacts the GM positively inducing production of 0.3 mg/kg of beneficial butyric acid. CHIC = Chitosan commercial; CHIW = Chitosan from seafood waste; FOS = Fructooligosaccharides; HPMC = Hydroxypropyl-methylcellulose; Red dotted line = Baseline values (values at the beginning of colonic GM fermentation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contributed to the increased production of detrimental catabolites from tryptophan and tyrosine fermentation, as Indole, skatole and p-Cresol. HPMC and CHIW produced higher amounts ofalso branched chain organic acids as Butanoic acid, 2-methyl and Pentanoic acid, 4-methyl, which are derived products of unhealthy protein fermentation by non-beneficial GM populations, as *Clostridium* group I, *E. coli* or *C. aerofaciens* (Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020). Lastly, CHIC produced just little amount of Pentanoic acid, 4-methyl but produced the highest amount of beneficial Butanoic acid and Propanoic acid. In particular, Butanoic acid amount was approx. 2.5 times more the value at the red dotted line, *i.e.* the baseline of GM fermentation (Fig. 4a). To this outcome it can be predicted that CHIC can impact the GM positively inducing production of 0.3 mg/kg of beneficial Butanoic acid to the well-being of colon environment and host bioavailability. In contrast, the increased production of Pentanoic acid 4-methyl- and Butanoic acid 2-methyl- in response to CHIW fermentation could represent an issue as a side-effect of CHIW, in fact the biosynthesis of branched chain fatty acids, could lead to gut dysbiosis (Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020). FOS significantly increased the synthesis of all short-chain fatty acids, which strengthens the confirmation of its prebiotic function. Regarding harmful compounds, both CHIC, CHIW and FOS showed a decrease in them. In contrast, HPMC increased negative compounds such as p-Cresol and Indole, indicating a possible activation of tryptophan-decomposing bacteria and proteolysis, which could have a negative effect on gut health (Wang, Gibson, et al., 2020; Wang, Meng, et al., 2020). These results line up with previous studies showing that chitosan increases the production of SCFAs during microbial fermentation, specifically acetate, propionate, and butyrate (Wang, Gibson, et al., 2020). In addition, the results support the prebiotic potential of non-plant-derived chitosan, which can enhance SCFA biosynthesis and preferentially promote healthy GM (Guan & Feng, 2022).

3.8. Multi-omics of in vitro outputs of modulations of metabolites and microbes by chitosan samples

Non-parametric correlation analysis was performed using the Spearman rank method between matrices of normalized data from each

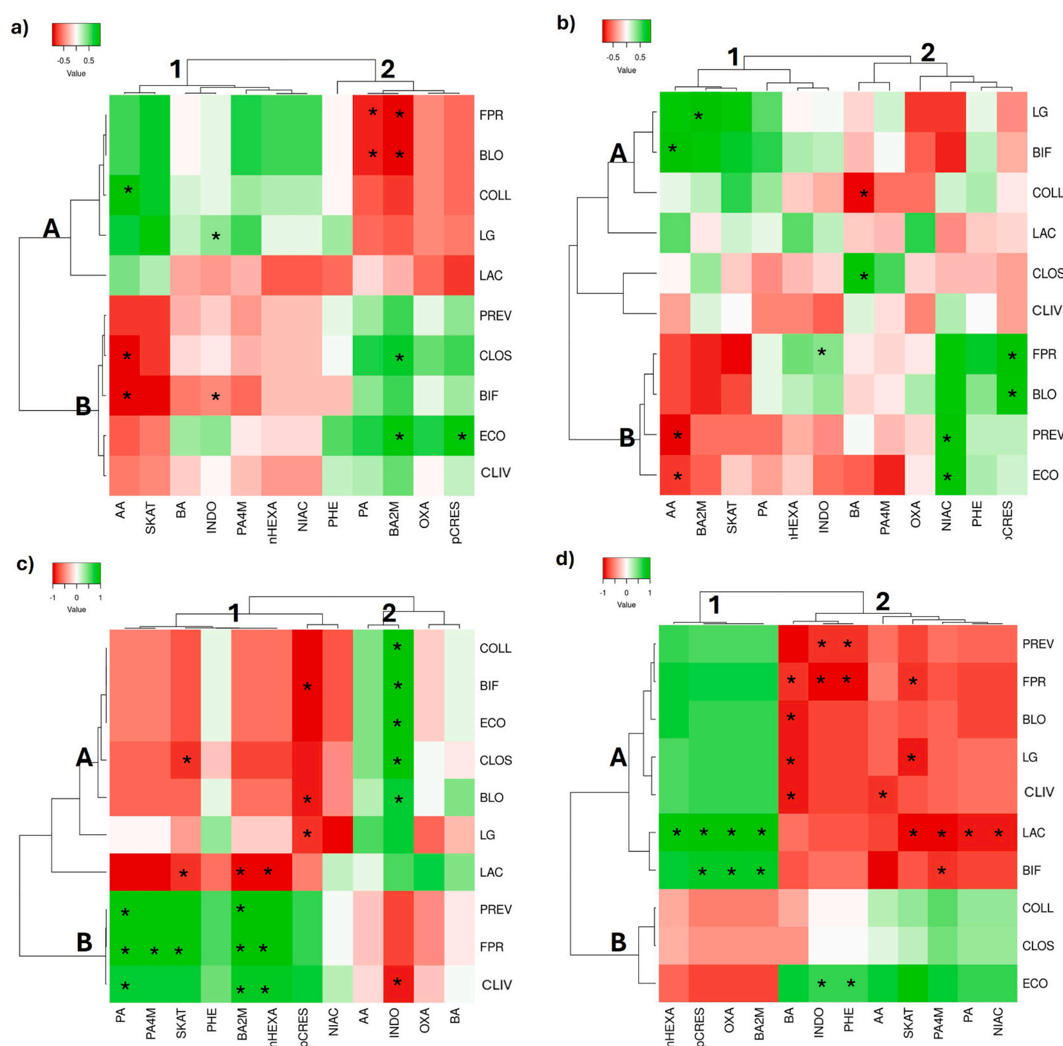

Fig. 5. Spearman-rank correlation heatmaps for bacteria and metabolites. (a) Colonic fermentation of Chitosan standard (CHIC); (b) Colonic fermentation of chitosan from seafood waste (CHIW); (c) Colonic fermentation of Fructooligosaccharides (FOS); (d) Colonic fermentation of Hydroxypropyl-methylcellulose (HPMC). Values were taken from shifts at the endpoint of colonic fermentation. Two-way heatmaps with dendrograms and clusterization without order scale. A, B, 1 and 2 indicate the clusters. AA = Acetic acid*;* SKAT = skatole; BA = Butanoic acid; INDO = Indole; PA4M = Pentanoic acid,4-mehtyl; nHEXA = n-Hexadecanoic acid; NIAC = Niacinamide; PHE = Phenol; PA = Propionic acid; BA2M = Butanoic acid, 2-methyl; OXA = Oxalic acid; pCRES = p-Cresol; CLIV = *Clostridium* group IV; ECO = *Escherichia coli*; BIF = *Bifodobacteriaceae*; CLOS = *Clostridium* group I; PREV = *Prevotella spp.*; LAC = *Lactobacillales*; LG = *Lactobacillus gasseri*; COLL = *Collinsella aerofacients*; BLO = *Bifidobacterium longum*; FPR = *Faecalibacterium prausnitzii*. Asterisks indicate significance of correlations as reported in supplemental material (Table S6).

tested food texturizers, including 12 significant volatile organic compounds (VOCs) and 10 significant bacterial taxa (ANOVA $p < 0.05$). Results were visualized with a two-way joining heatmap using HeatmappeR (Babicki et al., 2016), focusing on microbe-metabolite interactions (Fig. 5 and Table S6). In the CHIC fermentation dataset (Fig. 5a), four main variable clusters were identified. Cluster A1 showed strong positive correlations, particularly between *Lactobacillus gasseri* and acetic acid production, and *C. aerofaciens* and skatole. These findings are consistent with literature as it was reported an increased SCFA production, particularly acetate, in the presence of chitin-glucan substrates supporting *Lactobacillus spp.* activity (Marzorati et al., 2017).

Cluster A2 included negative correlations involving *Bifidobacterium longum* and *F. prausnitzii versus* branched-chain fatty acids such as 2-methyl butanoic acid, suggesting a protective effect against proteolytic fermentation. Similar antagonism between beneficial taxa and protein fermentation by-products has been observed in studies assessing dietary fiber fermentation (Louis et al., 2014). Additionally, *Lactobacillaceae* showed negative correlation with p-cresol, a phenolic compound linked to epithelial damage and dysbiosis (Smith & Macfarlane, 1996).

Cluster B1 revealed negative correlations between *Bifidobacteriaceae* and skatole, and between *Clostridium* group I and acetic acid. Cluster B2 highlighted positive correlations between *E. coli*, *Clostridium* group I, and toxic compounds such as p-cresol and branched-chain fatty acids, supporting recent findings reporting that synthetic texturizers can enrich opportunistic taxa producing deleterious metabolites (Bevilacqua et al., 2024).

For the CHIW dataset (Fig. 5b), correlations appeared more diffuse, but two minor clusters were evident. Cluster A1 showed positive associations of L. *gasseri* and *Bifidobacteriaceae* with acetic and propanoic acids, reinforcing the observations from CHIC and consistent with the known prebiotic effects of chitosan-derived oligosaccharides (Jeon, 2001). Interestingly, also *C. aerofaciens* correlated with skatole, suggesting a substrate-independent trait for the fermentation profile of this taxon. Cluster B1 demonstrated significant negative correlations of *F. prausnitzii* and *B. longum* with skatole and branched-chain fatty acids, again aligning with the notion that chitosan supports saccharolytic over proteolytic pathways.

Of particular note, both *B. longum* and *F. prausnitzii* showed significant positive associations with niacinamide production, a compound with emerging roles in host energy metabolism and immune regulation (Yoshikawa et al., 2020). This novel link suggests that CHIW may stimulate the capacity of the GM to generate host-beneficial metabolites beyond SCFAs, a feature not commonly observed with conventional texturizers. Considering colonic fermentations of FOS (Fig. 5c) among the results obtained, it is interesting to mention that *Lactobacillaceae* had negative correlations with skatole and Butanoic acid, 2-methyl and *Lactobacillus gasseri* with Aceti acid, while in the dataset of colonic fermentation of HPMC, the positive correlations among *E. coli* and Phenol and skatole were also noted (Fig. 5d).

Taken together, these results support the hypothesis that chitosan-based texturizers, particularly CHIW, foster a beneficial ecological and metabolic balance in the GM *in vitro*, contrasting with the dysbiotic signatures commonly reported for synthetic compounds. The microbial-metabolite correlation patterns observed agree with multiple studies that highlight the role of dietary substrates in steering microbial metabolism toward health-promoting outputs (Morrison & Preston, 2016).

3.9. Multi-omics of in vitro outputs with technological features of chitosan samples

Non-parametric correlation analysis was made by Spearman rank of two list matrices from specific normalized datasets of each given food texturizers, including 11 significant VOCs and bacterial variables and four technological variables (ANOVA $p < 0.05$), and visualized by two-way joining heatmap with HeatmappeR (Babicki et al., 2016) (Fig. 6 and Table S7). Correlations were visualized with hierarchical scale order by column among values of changes and shifts of VOCs and bacteria at the end point of colonic fermentation and technological conditions at the beginning of digestion, with the intention to evidence the technological feature that contributed more on GM modulation. Technological correlations for CHIC (Fig. 6a) and CHIW (Fig. 6b) were distributed and quantified differently, although were mainly assigned to Diacylation Degree (DD) variable. Among the correlations for CHIC, DD was linked to four health-related outputs explaining its beneficial impact on GM, namely: i) the capacity to foster beneficial bacteria, as lactobacilli, ii) *Clostridium* group IV and iii) bifidobacteria and iv) the reduction of detrimental p-Cresol. Minorly, among the correlations for CHIW, DD was linked to three health-related outputs, namely: the capacity to foster

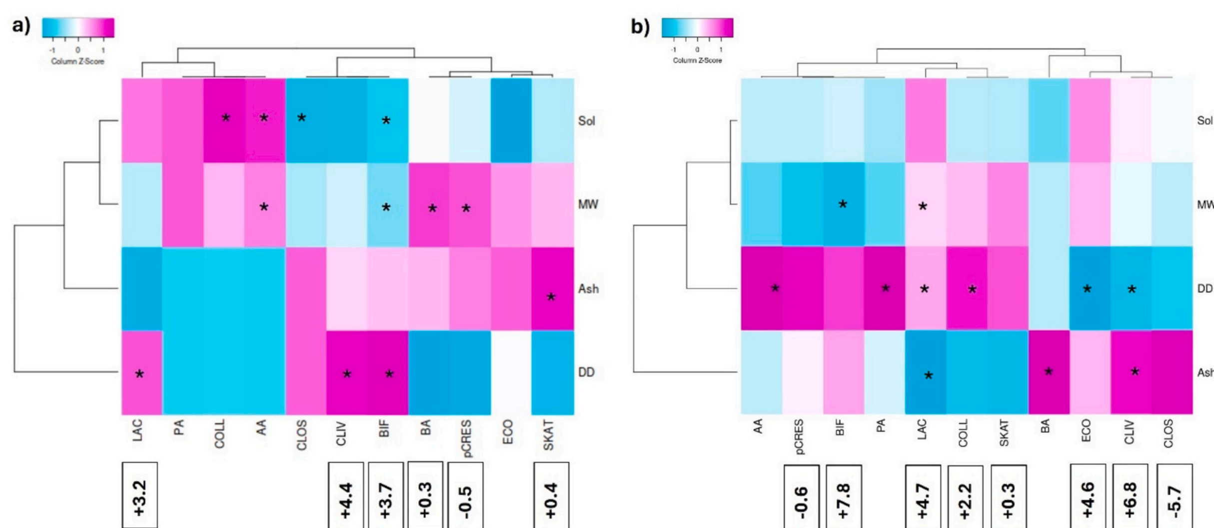

Fig. 6. Spearman rank correlation for technological features of chitosan samples. (a) Chitosan standard (CHIC); (b) chitosan from seafood waste (CHIW). Correlations of endpoint shifts of bacterial groups and metabolites against technological features of chitosan samples. Heatmaps are made by column-order and with dendrograms and clusterization. Labels with values stand for $Log_2(F/C)$ for shifts of bacteria and EP-BL difference for changes in metabolites concentration. LAC = *Lactobacillaceae*; PA = Propionic acid; COLL = *Collinsella aerofaciens*; AA = Acetic Acid; CLOS = *Clostridium* group I*;* CLIV = *Clostridium* group IV; BIF = *Bifidobacteriaceae*; BA = Butanoic acid; pCRES = p-Cresol; ECO = *Escherichia coli*; SKAT = skatole; DD = Degrees of Deacetylation; MW = Molecular weight; Sol = Solubility. Asterisks indicate significance of correlations as reported in supplemental material (Table S7).

beneficial i) bifidobacteria and ii) lactobacilli and iii) the inhibition of *Clostridium* group I. Other interesting insights for both chitosan samples are that solubility was linked to increased abundance of lactobacilli and that the effect of different MW has no acumen. Degree of deacetylation in chitosan refers to the amount of acetyl groups removed from its structure, leaving behind free amino groups (Yan et al., 2021). In line with our findings, it has been reported that while a DD higher than 90% can reduce the abundance of some beneficial bacteria, a DD lower than 80% can foster their growth and in particular that of lactobacilli (Guan & Feng, 2022). Considering solubility, it is reported that while chitosan is not a solvent for lactobacilli, it can interact with them, particularly in acidic conditions and can foster the growth of lactobacilli and in particular that of *L. plantarum* (Shahriari et al., 2024).

4. Conclusion

Dietary xenobiotics, including additives and contaminants, can disrupt the GM and contribute to metabolic or inflammatory intestinal disorders. The approach *in vitro* suggested in this work would present a case study responding to the EFSA expectation of robust and concrete approach of research and innovation expected in food safety. In fact, we demonstrated that the MICODE approach may be adopted by public and private stakeholders as a new *in vitro* approach since it deals with the EFSA and FAO needs of: a) studying of chemical-induced dysbiosis *via* robust *in vitro* methods identifying harmful metabolites and pathways for risk assessment; b) developing biomarkers of microbiome-related effects of dietary xenobiotics exposure.

Thus far, this study provided mechanistic insight into ecological and metabolic effects of chitosan and other texturizers on human GM. This work expands the knowledge over *in vitro* science of GM exposure to food additives, giving new insights as: i) absolute quantifications of the GM modulation of responder microbial groups; ii) characterization of the volatilome of the simulated colon niche after exposure to food additives; and iii) correlations among quantity values of microbes and metabolites and among microbial features and technological features. Additionally, in this work we have proved that MICODE is an *in vitro* model that meets the demands indicated by regulatory agencies (*i.e.* EFSA, FAO and FDA) necessary to approach contemporary RA protocols on food additives toward the GM, without using living animals or human subjects (FAO, 2025; Turck et al., 2024; US Food and Drug Administration, 2024). This work assesses and compares a novel type of artisanal-made chitosan from regional seafood wastes, as the carapace of *S. mantis*, in comparison to similar synthetic food additives, as synthetic chitosan and HPMC, confirming that the system works to test food ingredients prototypes. The RA toward the GM that we proposed gives also focus to byproducts particularly sensible to microbiological contamination and possibly vectors of undesired and non-human microbes, as important food safety traits fronting the valorization of food industrial byproducts. Both CHIC and CHIW showed similar beneficial effects on GM composition by significantly stimulating several probiotic and commensal populations, including *Lactobacillaceae*, *Bifidobacteriaceae*, *Bifidobacterium longum*, and *Clostridium* group IV. This result is particularly interesting, since CHIW was obtained by by-products of mantis shrimp processing, indicating an interesting strategy for the full valorization of this small crustacean. Additionally, CHIC promoted the production of health-related microbial metabolites such as butyric and propionic acids, which are linked to colonocyte health, immune modulation, and gut homeostasis. Some harmful volatile compounds were associated with either chitosan-derived substrates, as the production of branched chain fatty acids and skatole, for which ulterior safety assessments should be considered.

FOS, as expected, confirmed its prebiotic role, while HPMC presented some microbiological risk with limited functional benefit, raising questions about its continued use as a safe food additive. Additionally, multi-omics analyses corroborated our results saying that chitosan-based ingredients, particularly CHIC, modulate GM toward a beneficial ecological and metabolic profile, enhancing probiotic populations and suppressing harmful metabolites such as skatole. Technological characterization further revealed that the degree of deacetylation (DD) plays a pivotal role in shaping microbial responses, with intermediate DD values promoting bifidobacteria, lactobacilli and reducing toxic outputs like p-Cresol. Solubility also emerged as a favorable trait, notably associated with increased abundance of lactobacilli, while molecular weight showed negligible influence.

These findings highlight the relevance of structural optimization in designing GM-friendly texturizers and contribute to integrating data on GM in food safety RA of an alternative source of chitosan evidencing also some health benefits in comparison to synthetic texturizers as HPMC largely used. Chitosan thus represents a promising alternative to synthetic texturizers with potential applications in functional food development. The use of the MICODE *in vitro* model allowed for a robust, reproducible, and ethically sustainable platform to predict microbial shifts and metabolic output in response to complex substrates. The results presented are based on targeted qPCR and chromatographic techniques and could sound as a limitation of robustness, although this methodological choice was deliberate, as these techniques are widely available in most laboratories, cost- and time-effective, and highly reproducible. Notwithstanding, the future perspectives to strengthen and deepen the findings of this present work are to study the microbiome by metagenomic and transcriptomic means by NGS. While *in vitro* data cannot fully substitute *in vivo* evidence, they provide valuable insights that can guide ingredient development and safety assessment. In that direction, recently, a large consortium of scientists is working to the tentative to give guidelines and to promote the use of *in vitro* GM models (CA23110 INFOGUT https://www.cost.eu/actions/CA23110/).

Future validation in human trials will be critical to confirm these findings and translate them into regulatory and dietary practice.

CRediT authorship contribution statement

Davide Addazii: Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Ana Cristina De Aguiar Saldanha Pinheiro:** Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Lorenzo Nissen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Tappi:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Alessandra Bordoni:** Writing – original draft, Project administration, Investigation, Conceptualization. **Pietro Rocculi:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization. **Andrea Gianotti:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Conceptualization.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Institutional review board statement

The study was conducted according to the guidelines of the bioethical Committee required at the University of Bologna.

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Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.148927.

Data availability

Data will be made available on request.

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