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Influence of free and immobilized chitosan on a defined human gut microbial ecosystem

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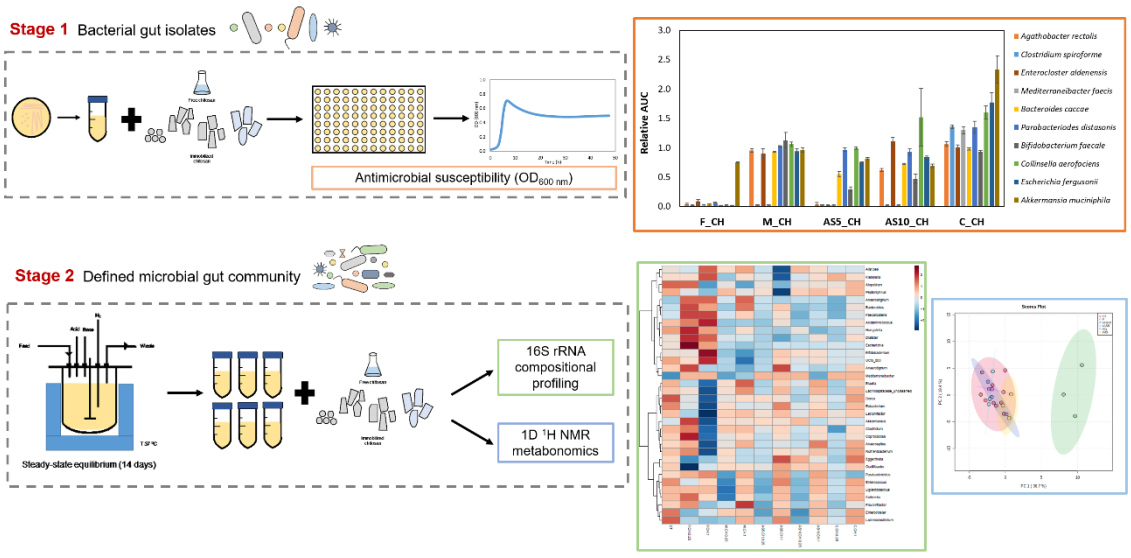
Graphical abstract



Highlights

- Impact of free/immobilized chitosan on human gut microbiota ecosystem was studied
- Individual gut isolates showed varied susceptibility to free/immobilized chitosan
- Ecosystem structure, viability and function was mainly altered by free chitosan
- Immobilization mitigated the impact of chitosan on microbiota composition
- Chitosan caused a decrease in the ratio of Firmicutes to Bacteroidetes

Abbreviations

Analisis of variance (ANOVA), amplicon sequencing variant (ASV), 10 µm-amorphous silica particles (AS10), 5 µm-amorphous silica particles (AS5), area under the curve (AUC), branched fatty acid (BCFAs), cellulose particles (C), chitosan (CH), chitosan oligosaccharides (COS), *N*-cetyltrimethylammonium bromide (CTABr), 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d6), free form (F), 3-(triethoxysilyl)propyl isocyanate (ISO), MCM-41 silica microparticles (M), 3-(triethoxysilyl)propyl isocyanate (ISO), mesoporous silica particles (M), principal components analysis (PCA), propidium monoazide (PMA), peptone yeast extract glucose broth (PYGB), short chain fatty acid (SCFAs), triethanolamine ($TEAH_3$), tetraethylorthosilicate (TEOS).

Abstract

In this work, the influence of different forms of presentation of chitosan in the human gut microbiota with a defined bacterial community was evaluated. First, the susceptibility of individual gut bacterial isolates against chitosan was studied within a concentration range between 0.125-1 mg/mL. Then, the impact of chitosan (0.25 and 1 mg/mL) on a defined human gut microbial ecosystem was studied by metagenomic and metabonomic analyses. The results showed that chitosan in its free form had a high impact on individual isolates with a minimum inhibitory concentration below 1 mg/mL for most of the strains studied. In comparison, chitosan immobilized in the different carriers displayed a diverse effect on gut microbiota. The most susceptible strains were *Agathobacter rectalis* strain 16-6-I 1 FAA*, Clostridium spiroforme* strain 16-6-I 21 FAA and *Mediterraneibacter faecis* strain 16-6-I 30 FAA. The impact of the different modes of presentation of chitosan was strain-specific and species-specific when compared to results obtained from analysis of other strains within the genera *Agathobacter*, *Clostridium* and *Mediterraneibacter,* and therefore a study using a defined ecosystem was needed to extrapolate the results. Significant decreases in defined community richness and diversity and changes in metabolic profile were observed after exposure to free chitosan. Free chitosan produced significant reductions in the abundance of the genera *Lachnoclostridium*, *Anaerotignum*, *Blautia*, *Enterococcus*, *Eubacterium* and *Ruthenibacterium* together with a slight decrease of the production of SCFAs, among other fermentation by-products. The immobilized chitosan significantly alleviated the impact caused by the antimicrobial polymer and significantly increased the relative abundance of the Bacteroidetes phylum compared to free chitosan. These results suggest the significance of assessing the impact of new ingredients and materials included in food on the human gut microbiota with models that simulate the gastrointestinal environment, such as *in vitro* bioreactor systems.

Keywords: antimicrobial polymer; bacterial gut isolates; Bacteroidetes; covalent immobilization; defined gut microbial community; food preservatives.

1. Introduction

Chitosan is a cationic amino polysaccharide, composed of randomly distributed β-(1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine, with a variety of biological activities. It is used as a food additive, in material packaging and as a dietary supplement (Wang et al., 2021). Chitosan can be applied to foods as a preservative, thickener, decolorant, flocculant and/or stabilizer (Wang, Xue, & Mao, 2020). Chitosan has antimicrobial activity against bacteria and fungi mainly as a result of the interaction between the positive charges on the amine group of the glucosamine monomer in chitosan with the negatively charged microbial cell membrane (Yan, Zhang, Cao, Feng, & Li, 2020). The antimicrobial activity depends on the molecular weight of chitosan that, in turn, determines its solubility. Chitosan is an insoluble fiber at neutral and alkaline pH values, and this property limits its application as a food preservative (Qin et al., 2006). It is therefore normally dissolved in an acidic medium, which also affects the antimicrobial activity (Wang et al., 2020). Different derivatives of chitosan can be obtained with the help of chemical modifications, for example, chitosan oligosaccharides (COS). COS are a mixture of oligomers of chitosan with a low molecular weight (<5,000 Da) that are soluble in water, although their antimicrobial potential is not as great as for chitosan alone (Šimůnek, Brandysová, Koppová, & Šimůnek, 2012; Yan et al., 2020).

Chitosan and its derivatives are non-digestible fibers also used as dietary supplements with functions that include fat binding (Gulbake & Jain, 2012) and prebiotic substrate (Tang et al., 2020). The gut microbiota includes component microbes which possess the genetic potential to hydrolyze non-digestible polysaccharides such as chitosan (Yan et al., 2020), for example chitinolytic bacteria that excrete enzymes with chitinase, chitosanase, chitobiosidase and *N*-acetyl-β-glucosaminidase activities capable of degrading chitosan and its derivatives (Gulbake & Jain, 2012; Šimůnek, Tishchenko, & Koppová, 2008). Microbial fermentation can also convert the polysaccharide residues to beneficial metabolites such as short-chain fatty acids (SCFAs) (Vinke, El Aidy, & van Dijk, 2017). Therefore, chitosan and its derivatives have

been postulated to have the ability to impact human gut microbiota disorders by modulating the microbiota composition and metabolic function (Tang et al., 2020).

Given the limited solubility of chitosan, a few studies have reported the influence of chitosan on human gut microbiota. Chitosan variants with different molecular weight and deacetylation degrees produced a significant impact on fecal microbiota samples resulting in a decrease in key genera such as *Bacteroides*, *Bifidobacterium*, *Clostridium* and *Lactobacillus* (Mateos-Aparicio, Mengíbar, & Heras, 2016; Vernazza, Gibson, & Rastall, 2005). On the other hand, further studies have focused on the influence of COS on the gut microbiota *in vitro* and *in vivo* (Shang et al., 2017; Šimůnek et al., 2012; Zhang, Jiao, Wang, & Du, 2018), showing a lower antimicrobial activity of COS compared to chitosan (Šimůnek et al., 2012), and indicating the influence of COS on microbiota composition and metabolic function (Yan et al., 2020). COS caused a reduction of species from the phylum Proteobacteria, an increase in SCFA-producing genera such as *Akkermansia* and *Blautia* and a variable influence on probiotic genera such as *Bifidobacterium* and *Lactobacillus* (Shang et al., 2017; Q. Wang et al., 2020; Zhang et al., 2018).

According to previous research, the use of chitosan as food additive or supplement might influence human gut microbiota composition, resulting in a dietary intervention with notable effects on host health (Vinke et al., 2017; You et al., 2017). Therefore, there is a need to understand the potential interactions between chitosan and gut microbiota. Most of the literature related to this has been focused on COS that are soluble in water and which have low antimicrobial activity. In this work, the covalent immobilization (Ruiz-Rico & Barat, 2021) of chitosan onto the surface of different siliceous and cellulosic supports as a strategy to improve the utilisation of chitosan was explored. For this, the exposure of the gut microbes to chitosan was performed using different modes of presentation of the polymer to evaluate the influence of the carrier on the impact of chitosan on the gut microbiota. The impact of chitosan was assessed *in vitro* in 2 settings: (i) susceptibility tests of chitosan on selected human gut microbiota members, and (ii) assessment of

community perturbation (through metagenomics and metabonomics analyses) by chitosan using a defined model gut microbial ecosystem supported in a chemostat bioreactor modeling conditions of the human colon.

2. Materials and methods

2.1. Reagents

For the preparation of the different forms of presentation of chitosan the following reagents were used. Low molecular weight chitosan (CH) (50,000-190,000 Da), 10-µm amorphous silica microparticles (AS10), microcrystalline cellulose particles (C), *N*-cetyltrimethylammonium bromide (CTABr), tetraethylorthosilicate (TEOS), triethanolamine ($TEAH_3$), 3-(triethoxysilyl)propyl isocyanate (ISO), acetic acid (≥99%) and NaOH were provided by Sigma-Aldrich (Madrid, Spain). 5-µm amorphous silica microparticles (AS5) were supplied by Silysiamont (Milano, Italy). Ethanol absolute was acquired from Scharlab (Barcelona, Spain).

For evaluating the impact of chitosan on human microbiota, the following growth media were used. Modified Peptone Yeast extract Glucose Broth (PYGB) was prepared according to the protocol described in Table S1, supplementary information. Fastidious anaerobe agar was supplied by Neogen Corporation (Lansing, USA) and defibrinated sheep's blood was provided by Hemostat Laboratories (Dixon, USA). Chemostat medium was prepared according to Table S2, supplementary information. For analyses of ecosystems, propidium monoazide (PMA) was acquired from Biotium (Fremont, USA), while QIAamp PowerFecal Pro DNA Kit was provided by Qiagen Sciences (Germantown, USA) and Nextera XT Index V2 sequences were provided by Illumina Inc. (San Diego, USA). gDNA template in Invitrogen Platinum PCR SuperMix High Fidelity and Invitrogen PureLink PCR purification kit were supplied by Life Technologies Inc. (Burlington, Canada). 5 mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d6) containing 0.1% sodium azide was acquired from Chenomx Inc. (Edmonton, Canada).

2.2. Synthesis of chitosan-coated supports

The synthesis of mesoporous silica microparticles MCM-41 (M) was carried out following the 'atrane route' using CTABr as the structure-directing agent. The molar ratio of the reagents was 7 $TEAH_3$: 2 TEOS: 0.52 CTABr: 0.5 NaOH: 180 H_2O (Verdú et al., 2020) (see Supplementary Information for details).

Once M support was obtained, chitosan-coated (CH-coated) supports were prepared by surface silanization of the supports (M, AS5, AS10 and C) with an organosilane (ISO) to decorate the external surface with isocyanate moieties and covalent immobilization of the natural antimicrobial polymer chitosan by urea bonding between the isocyanate residues on the surface's support and the amino group of chitosan (Scheme S1, supplementary information) (Polo et al., 2017). The details of the immobilization procedure can be found in Supplementary Information, obtaining the CH-coated supports (**M_CH**, **AS5_CH**, **AS10_CH** and **C_CH**).

2.3. Characterization of chitosan-coated supports

The bare and CH-coated silica microparticles were characterized by standard techniques to determine their particle size, surface charge and degree of functionalization. Particle size distribution was analyzed in deionized water using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) with previous sonication. The Mie theory was applied to determine particle size using a refractive index of 1.45 and an absorption index of 0.1 for M particles and 0.001 for AS5, AS10 and C supports. Surface charge was determined by zeta potential analysis using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples were suspended in deionized water (1 mg/mL) and sonicated before being measured to prevent the agglomeration of microparticles. The electrophoretic mobility measurements were converted into zeta potential values by the Smoluchowsky mathematical model. Degree of functionalization was established by elemental analysis for C, H and N in a

CHNOS Vario EL III model (Elemental Analyses System GMHB, Germany). All the analyses were conducted in triplicate. Results were statistically analyzed by an analysis of variance (ANOVA) using Statgraphics 18 (Statpoint Technologies, Inc., Warrenton, USA).

2.4. Bacterial gut isolates

In a first step, the impact of free (**F_CH**) or immobilized chitosan (**M_CH**, **AS5_CH**, **AS10_CH** and **C_CH**) was assessed on the growth of bacterial gut isolates of commensal species representative of the main phyla of the human gut microbiota. The bacterial isolates were obtained from a collection of previously-isolated strains derived from stool samples from different donors. Initially, 10 bacterial strains (Table S3, supplementary information) of a defined gut microbiota community from a healthy donor (donor A) were used for exposure studies with single strains (details of defined ecosystem in Section 2.6). Once the strains that were most susceptible to chitosan were established, additional growth curve assays were performed with taxonomically closely related isolates from Donor A or from other donors (B, C and D) (see Table S4, supplementary information, for details).

2.5. Susceptibility of bacterial gut isolates against free and immobilized chitosan

Antimicrobial susceptibility assays were performed by monitoring growth curves of the bacterial gut isolates in response to a range of concentrations (0.125, 0.25, 0.5 and 1 mg/mL) of free or immobilized chitosan. The range of concentrations tested was chosen according to previous *in vitro* studies with free chitosan against similar non-pathogenic gut microbes (Šimůnek et al., 2012; Šimůnek, Koppová, Filip, Tishchenko, & Bełżecki, 2010). For the preparation of **F_CH** stock solution (2%), chitosan was dissolved in acetic acid 1% under stirring at 50ºC for 24 h. To evaluate equivalent concentrations of immobilized chitosan, the amount of CH-coated supports needed was calculated based

on the content of organic matter grafted on the surface of the supports, according to elemental analysis.

The bacterial growth was monitored using a 96-well plate. For **F_CH**, microplate wells were filled with the required volume of PYGB and chitosan stock solution to obtain 190 µL-wells with the target concentrations of chitosan. Then, 10 µL of the inoculum (see Supplementary Information for details) was added to each of the wells and the microplate was incubated anaerobically at 37°C in a plate reader (Epoch 2 Microplate Spectrophotometer, BioTek, USA) with continuous agitation.

For the CH-coated supports, the assay protocol was modified to include a previous exposure of the strains to the supports in conical tubes. The corresponding amount of particles were suspended in conical tubes with 2.5 mL of PYGB, the inoculum was added and the tubes were wrapped and transferred to gas jars. The conical tubes were incubated anaerobically with orbital stirring at 37ºC for 24 h within a shaking incubator (Minitron, Infors HT, Switzerland). After the contact period, samples were centrifuged at 1500 rpm for 2 min to sediment the supports. Then, 10 µL of the previously-exposed supernatant (containing bacterial cells) was placed into fresh PYGB in a 96-well plate and the growth was further monitored throughout incubation at 37°C for 48 h under anaerobic conditions (García-Ríos, Ruiz-Rico, Guillamón, Pérez-Esteve, & Barat, 2018).

For each experimental series, control inoculated wells without chitosan were included to monitor the growth of the strain in absence of treatment, and non-inoculated wells (with or without chitosan) were also included to subtract of any noise signal. In addition, inoculated samples with added solvent (acetic acid 1%) and non-modified supports were included to assess any potential growth effects their presence may have had on each strain. All experiments were carried out in triplicate.

Growth measurements (OD_{600}) were automatically recorded every 30 min over a 48 h-period. The area under the OD_{600} *vs.* the time curve (AUC) from average data was calculated, through the use of the R package growthcurver (Sprouffske & Wagner, 2016). Relative AUC values were calculated in accordance to control condition without

treatment. Significance was determined by one-way and multifactor analyses of variance (ANOVA) using Statgraphics 18.

2.6. Defined microbial gut community

Once the susceptibility of isolates was established individually, the impact of the different forms of presentation of chitosan was evaluated using a defined bacterial community as a complex ecosystem model. A 124-strain, 69-species defined bacterial community from a collection of previously-isolated strains derived from a stool sample of a healthy donor (Donor A) was used as a microbiota model (Petrof et al., 2013).

The ecosystem was cultured *in vitro* within a continuously feeding bioreactor (chemostat) which allows microbial communities derived from fresh feces to reach an equilibrium that resembles the *in vivo* distal gut community from which they originate. 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 added to the degassed medium within two 500-mL Multifors bioreactor vessels (Infors HT, Switzerland). 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 of 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. 2-mL samples (x3) were taken daily to analyze the evolution of the ecosystem. The samples were stored at −80°C until use.

2.7. Exposure of defined gut community to free and immobilized chitosan

Exposure to free or immobilized chitosan was carried out by incubating the obtained ecosystem in the presence of the different presentation modes of chitosan in discontinuous culture (Dudefoi, Moniz, Allen-Vercoe, Ropers, & Walker, 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 antimicrobial by adding **F_CH** or CH-coated supports to achieve final concentrations of 0.25 and 1 mg/mL. Then, the inoculated tubes were sealed and transferred to gas jars and incubated anaerobically at 37ºC for 48 h under orbital shaking. Untreated samples and samples exposed to the solvent 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 -80ºC until use. The evaluation of the impact of chitosan on microbiota was carried out by monitoring the composition, viability, and metabolic profile of the ecosystem.

2.8. 16S rRNA compositional profiling

Dynamic changes in the gut microbial community were analyzed by 16S RNA gene sequencing. In order to profile only living bacteria, PMA pretreatment was performed on the exposed chemostat samples to exclude DNA from bacteria with compromised membrane structure to subsequent amplification (Stinson, Keelan, & Payne, 2019). After PMA pretreatment, QIAamp PowerFecal Pro DNA Kit was utilized to extract gDNA from the PMA-treated samples. 16S rRNA gene libraries were prepped with 400 ng of Nextera XT Index V2 sequences plus standard V4 region primers and 2 µL of gDNA template in Invitrogen Platinum PCR SuperMix High Fidelity as a one-step PCR amplification. PCR products were purified using an Invitrogen PureLink PCR purification kit. 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 (Oliphant et al., 2020). Details of PMA pretreatment, PCR amplification and sequences processing can be found in supplementary information. Finally, statistical analyses were performed and graphs were plotted using MicrobiomeAnalyst (Chong, Liu, Zhou, & Xia, 2020).

2.9. 1D ^{1}H NMR metabonomics

The metabolic profile of the ecosystem was characterized by 1D ^{1}H NMR spectroscopy following the procedure described by (Ganobis et al., 2020). 540 µL of sterile-filtered cell-free samples were combined with 60 µL of the internal standard 5 mM DSS-d6 in deuterated water, containing 0.1% sodium azide. Samples were scanned on a Bruker Avance 600 MHz spectrometer, housed at the NMR Centre (Advanced Analysis Center, University of Guelph). Spectra files were processed using Chenomx NMR Suite 8.5 (Chenomx Inc., Canada). Details of scanning and spectra processing can be found in supplementary information. Statistical analyses were performed and graphs were plotted using MetaboAnalyst (Chong & Xia, 2018).

3. Results and discussion

3.1. Characterization of chitosan-coated supports

The four CH-coated supports prepared by covalent immobilization of chitosan on the surface of carriers were characterized by standard instrumental techniques. Table 1

shows the mean particle size, surface charge and degree of functionalization of the bare and CH-coated supports.

Table 1. Particle size ($d_{0.5}$), zeta potential (ζ) and degree of functionalization (α) of bare and chitosan-coated mesoporous silica (M), 5-µm amorphous silica (AS5), 10-µm amorphous silica (AS10) and cellulose (C) supports.

Support	Functionalization	$d_{0.5}$ (µm)	ζ (mV)	α (g organic matter/g solid)
M	Bare	0.67 ± 0.07^{ns}	-38.10 ± 0.75^{a}	n.m.
	Chitosan	0.68 ± 0.06^{ns}	17.08 ± 2.63^{b}	0.027
AS5	Bare	3.57 ± 0.41^{ns}	-26.10 ± 0.91^{a}	n.m.
	Chitosan	3.45 ± 0.22^{ns}	17.98 ± 0.74^{b}	0.043
AS10	Bare	8.00 ± 0.50^{ns}	-18.20 ± 4.73^{a}	n.m.
	Chitosan	7.37 ± 0.71^{ns}	11.30 ± 0.56^{b}	0.041
C	Bare	4.57 ± 0.40^{ns}	-13.83 ± 0.97^{a}	n.m.
	Chitosan	4.57 ± 0.09^{ns}	-5.17 ± 0.04^{b}	n.m.

n.m.: non-measured

Different superscripts denote differences among the silica supports ($p<0.05$), ns: non-significant

The particle size distribution was preserved after chitosan immobilization, with a mean particle size of approximately 0.7 µm for the smallest support (M), 3.5-4.6 µm for the medium-size carriers (AS5 and C), and 7.4-8 µm for the largest support (AS10). Regarding the surface charge, the zeta potential values show the significant changes in the surface charge after immobilization of chitosan. While the bare supports had negative zeta potential due to the deprotonated hydroxyl groups that are present on the surface of cellulosic and siliceous materials, the CH-coated carriers displayed positive zeta potential values due to the attachment of the positively charged polymer (Popat, Liu, Lu, & Qiao, 2012; Zhao et al., 2017). The cellulosic support presented a negative zeta

potential after immobilization, probably indicating the low yield of the grafting reaction in this carrier that could limit its antimicrobial properties. The degree of functionalization of the carriers was established according to the content of organic matter grafted on the surface of the supports. The use of amorphous silica supports (AS5 and AS10) resulted in a higher attachment of the chitosan-organosilane moieties, compared to the M support. The values of the degree of functionalization obtained were used to calculate the amount of the different solids required to evaluate the equivalent concentrations of the free and immobilized chitosan.

3.2. Bacterial gut isolates show a considerable variation in susceptibility to the different forms of presentation of chitosan

First, the susceptibility of 10 strains from the defined community (donor A) was studied against the different forms of administration of chitosan. Figure 1 shows the relative AUC for each the 10 microorganisms, compared to the control condition, for the highest concentration used in this study (1 mg/mL) of the free or immobilized chitosan. **F_CH** completely inhibited the growth of all the gut microbes tested, except *Akkermansia muciniphila* (strain 3 FMU) ($p<0.001$). Otherwise, the susceptibility of the strains tested after exposure to the different presentation forms of immobilized chitosan was diverse. Three general responses of the tested isolates to immobilized chitosan were seen: (i) no influence on microbial growth; (ii) bactericidal effect exhibited; or (iii) bacterial growth was improved.

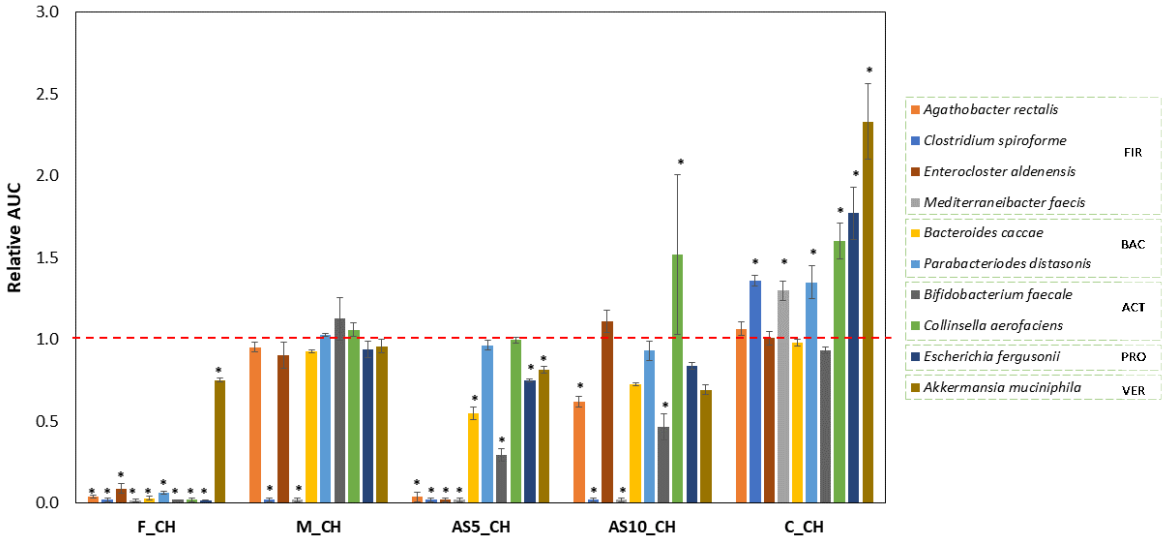


Figure 1. Relative AUC of the bacterial gut isolates of different phyla (FIR: Firmicutes, BAC: Bacteroidetes, Actinobacteria: ACT, Proteobacteria: PRO, and VER: Verrucomicrobia) after incubation with 1 mg/mL of chitosan (CH) administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C) (means and standard deviations, n=3). The values are relative compared with the control condition without treatment. (*) *p<0.001* indicates significant differences compared to the control.

The statistical analysis of the results by multifactorial ANOVA (Tables S5 and S6, supplementary information) confirmed the significant influence of the factors (strain and mode of presentation of chitosan) on the microbial growth (*p<0.001*), being the mode of presentation of the antimicrobial polymer the most significant factor on the microbial viability. In general, the most susceptible strains were *A. rectalis* strain 16-6-I 1 FAA, *C. spiroforme* strain 16-6-I 21 FAA and *M. faecis* strain 16-6-I 30 FAA, all of which are representative of Firmicutes, which in turn is generally the most abundant phylum in the gut microbiota. In contrast, the growth of the strains of other important phyla Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia was slightly affected by the biocidal polymer. In fact, *A. muciniphila* (strain 3 FMU) was not notably affected

by any of the modes of presentation of chitosan. The non-effect or even the promotion of the growth of this microorganism by chitosan has been recently described (Yan et al., 2020). *A. muciniphila* is a mucin-degrading bacterium commonly found in the intestinal mucus of human gut, representing 3–5% of the microbial community in humans. *A. muciniphila* is under consideration as a new probiotic because it has been positively associated with mucus thickness and intestinal barrier integrity and negatively associated with obesity, diabetes, inflammation, and metabolic disorders (Ottman, Geerlings, Aalvink, de Vos, & Belzer, 2017). This is related with the degradation of mucin that results in production of SCFAs that stimulates mucus turnover rate because they are used as preferable energy sources for the host epithelium which synthesize and secret mucin. As well, the breakdown products of mucin, such as oligosaccharides, sialic acid or sulfate, released by *A. muciniphila* can be used by other intestinal commensal bacteria located on the outer layer of intestinal mucus as a nutrient source and result in greater thickness of intestinal mucus (Yan et al., 2020; Zhou, 2017).

Regarding the immobilization supports, the use of amorphous silica particles (**AS5_CH** and **AS10_CH**) resulted in greater antimicrobial properties of chitosan than the use of **M_CH**. In contrast, **C_CH** support did not show antimicrobial properties at all and, for some microorganisms, growth was enhanced after exposure to **C_CH**, probably because these bacteria use it as a fermentation substrate with prebiotic function. The impact of solvent acetic acid (for free form) and bare supports (for immobilized forms) on microbial growth was also studied to ensure that the antimicrobial activity was only associated with chitosan. As can be seen in Figure S1, supplementary information, none of the conditions resulted in an inhibitory effect, although the growth curves obtained after exposure to non-coated cellulose support were, in some cases, different from the control group, which might corroborate the influence of the support on the impact on microbial growth.

The antimicrobial activity of **F_CH** was studied in the concentration range between 0.125-1 mg/mL (Figure S2, supplementary information). The Verrucomicrobia

representative species (*A. muciniphila* strain 3 FMU) was not susceptible to chitosan and its growth was even enhanced by low concentrations of this polymer. In contrast, **F_CH** was highly effective against the strains belonging to the phyla Firmicutes and Actinobacteria even using low concentrations of the antimicrobial, while the strains of the phyla Bacteroidetes and Proteobacteria were affected only by high concentrations. The antimicrobial activity of chitosan and COS against some commensal gut bacteria such as lactic acid bacteria and bifidobacteria has been previously reported in *in vitro* (Šimůnek et al., 2012) and *in vivo* studies (Zhang et al., 2018). *In vitro* studies of free chitosan with a similar molecular weight against gut bacterial isolates displayed minimum inhibitory concentrations in a similar range (0.1-0.5%) compared to this study for strains of the genera *Bifidobacterium*, *Bacteroides* or *Clostridium* (Lee, Park, Jung, & Shin, 2002; Šimůnek et al., 2012, 2010).

In addition, it should be considered that the inhibitory properties of **F_CH** are linked to the solvent used for its dispersion. The impact of the acetic acid solution can be seen in Figure S1, supplementary information, and although in itself it did not inhibit microbial growth, the antimicrobial activity of **F_CH** is probably influenced by an additive or synergistic effect of acetic acid (W. Wang et al., 2020). In contrast, the antimicrobial properties exhibited by the CH-coated supports are based solely on the inhibitory effect of the chitosan grafted on the surface of supports.

3.3. The impact of the different presentation forms of chitosan is strain-specific and species-specific

The effect of the different modes of administration of chitosan in a concentration range between 0.125-1 mg/mL of chitosan against the most susceptible strains (*A. rectalis* strain 16-6-I 1 FAA*, C. spiroforme* strain 16-6-I 21 FAA and *M. faecis* strain 16-6-I 30 FAA) is presented in Figure 2.

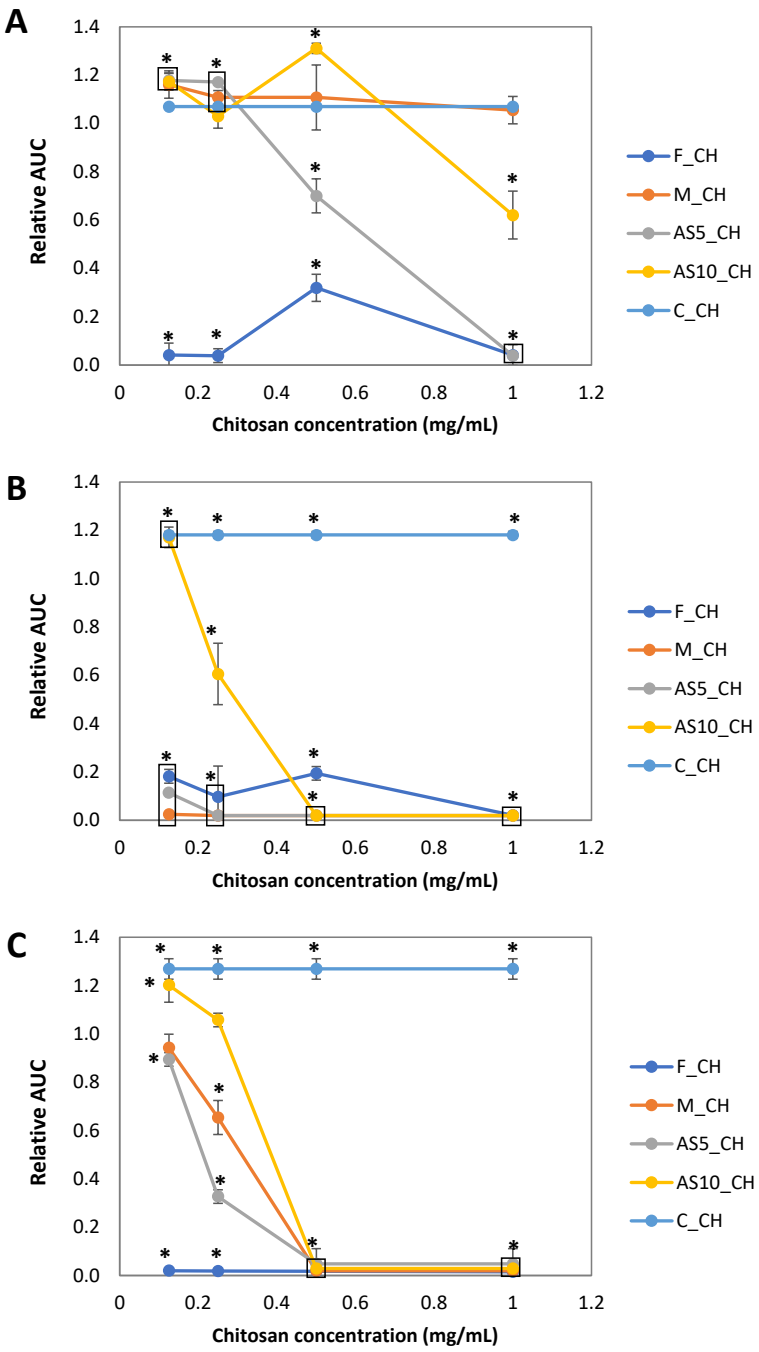


Figure 2. Relative AUC of *Agathobacter rectalis* strain 16-6-I 1 FAA (A), *Clostridium spiroforme* strain 16-6-I 21 FAA (B) and *Mediterraneibacter faecis* strain 16-6-I 30 FAA (C) after incubation with different concentrations of chitosan administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C) (means and standard deviations, n=3). (*) *p<0.001* indicates significant differences compared to the control.

A. rectalis (formerly *Eubacterium rectale)* strain 16-6-I 1 FAA (Fig. 2A) was completely inhibited by **F_CH** and **AS5_CH** using a concentration of 1 mg/mL (*p<0.001*). The growth of *C. spiroforme* strain 16-6-I 21 FAA (Fig. 2B) was fully inhibited by all the forms of presentation of chitosan, except **C_CH**, within the whole range of concentrations (0.125-1 mg/mL). In this case, the use of **M_CH** and **AS5_CH** were the most effective carriers of chitosan with a minimum inhibitory concentration below 0.25 mg/mL (*p<0.001*). Similarly, *M. faecis* (formerly *Ruminococcus faecis*) strain 16-6-I 30 FAA (Fig. 2C) was inhibited by **F_CH** and CH-coated silica supports, but in this case, the use of **F_CH** resulted in greater growth inhibition compared to the other administration forms (*p<0.001*). Total inhibition of the microorganism was achieved with 0.125 mg/mL of **F_CH**, while a concentration of 0.5 mg/mL was needed to eradicate *M. faecis* strain 16-6-I 30 FAA with the CH-coated silica carriers. In addition, Table S7, supplementary information, shows the statistical results of the multifactor ANOVA analysis confirming the significant influence of the factors (mode of presentation, concentration and their interaction) on the microbial growth.

The effect of free or immobilized chitosan on the growth of different strains within the genera *Agathobacter*, *Clostridium* and *Mediterraneibacter* present in the defined gut community (donor A) and isolates from stool samples obtained from other donors (donors B, C and D) are presented in Figure 3 and Table S8, supplementary information. The different modes of presentation of chitosan showed a strain-specific and species-specific impact and therefore a broad effect of chitosan on related species or genera cannot be stated. The strain and species variation in susceptibility to chitosan was also observed in the study of (Tsai & Hwang, 2004) evaluating the *in vitro* susceptibility of different *Lactobacillus* and *Bifidobacterium* probiotic species.

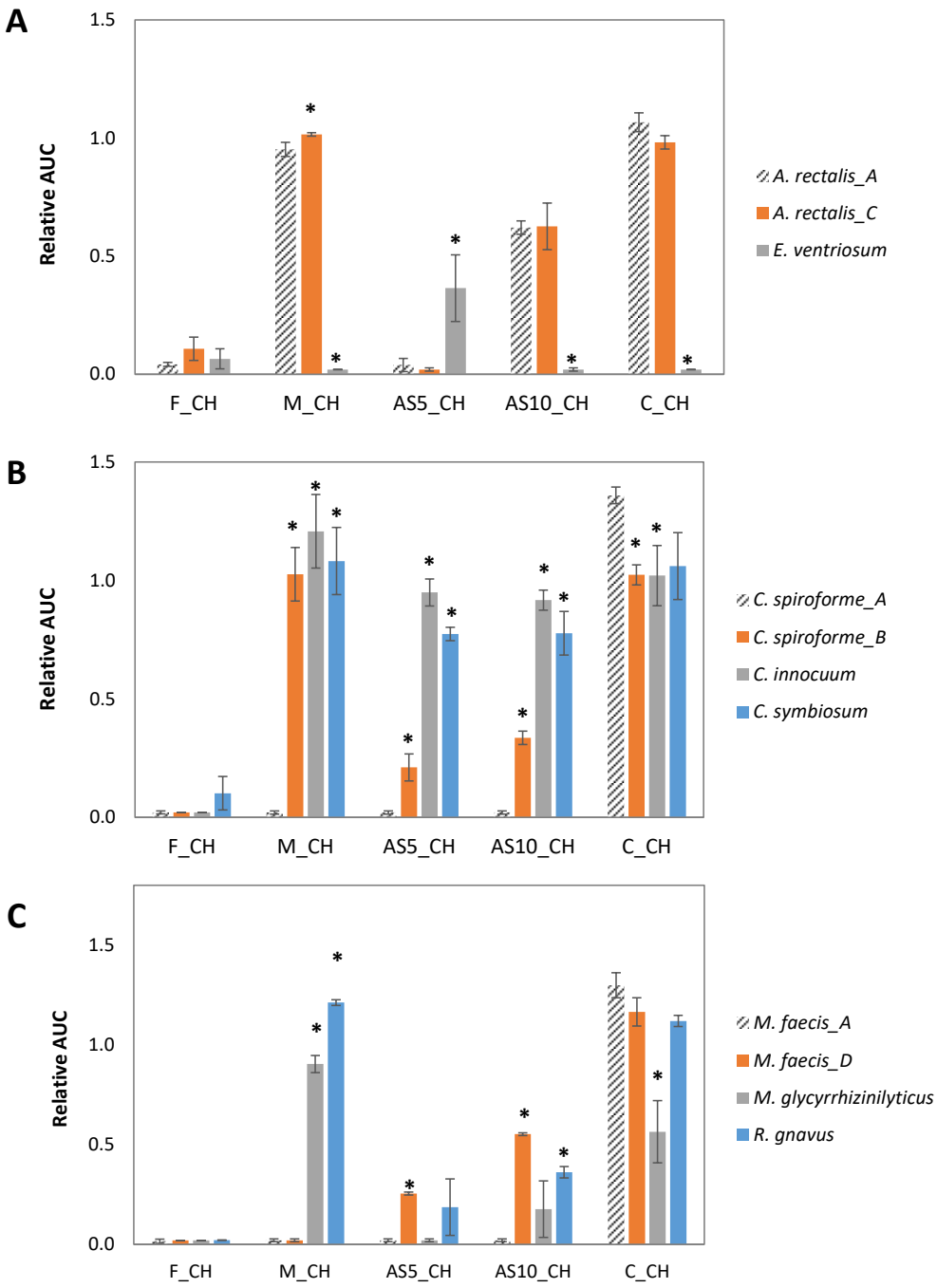


Figure 3. Relative AUC of *Agathobacter*-related species (A), *Clostridium* species (B) and *Mediterraneibacter*-related species (C) after incubation with 1 mg/mL chitosan administered in their free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C) (means and standard deviations, n=3). (*) *p<0.05* indicates significant differences compared to control strains (striped bars).

The impact of chitosan in *Agathobacter*-related strains (Fig. 3A) was similar to the reference strain (*A. rectalis* strain 16-6-I 1 FAA from donor A), although *E. ventriosum* strain 16-6-I 47 FAA (another member of the *Lachnospiraceae* family) was significantly more affected than the other strains ($p<0.05$). Both species are SCFAs-producing bacteria considered beneficial for health (Ji et al., 2022).

For the genus *Clostridium* (Fig. 3B), the microbial growth of the evaluated strains was statistically different than that of the reference strain (*C. spiroforme* strain 16-6-I 21 FAA from donor A) ($p<0.05$), with a lower inhibitory effect for *C. spiroforme* strain 58 TSA from donor B, *C. inocuum* (strain 16-6-S 16 LG) and *C. symbiosum* (strain 16-6-S 5 FAA). The strains tested form part of the main members of gut microbiota population *Clostridium* cluster XIVa (*C. symbiosum*), *Clostridium* cluster XVI (*C. innocuum*) and XVIII (*C. spiroforme*), which are considered beneficial species because of their reported anti-inflammatory properties (Goldstein, Citron, & Tyrrell, 2014; Van Den Abbeele et al., 2013). The effect of free chitosan against different pathogenic species of the genus *Clostridium*, such as *C. difficile* (Choi, Lee, Yu, & Chae, 2015) and *C. perfringens* (Chang, Chen, & Tsai, 2020), has been previously reported and is in accordance with our results. However, the *Clostridium* genus is recognized to be highly polyphyletic and thus representative species may not be closely related despite sharing a genus name. For this reason, it is not easy to extrapolate results from one *Clostridium* sp. to another.

For the genus *Mediterraneibacter* (Fig. 3C), *M. faecis* strain 16-6-I 30 FAA was generally more affected by chitosan treatment than the other strains evaluated ($p<0.05$). *M. faecis* is considered as beneficial species as a result of to its capacity to produce several SCFAs (Ye et al., 2021). Similarly, *M. glycyrrhizinilyticus* (previously classified as *Clostridium glycyrrhizinilyticum*) is a species belonging to the core gut microbiome group, *Clostridium* cluster XIVa (Wu et al., 2020). *R. gnavus* is a member of the core gut microbiota that degrades mucin and is able to use sialic acid as a sole carbon source. This likely provides *R. gnavus* with a competitive advantage and interestingly this genus

has been associated with various diseases related to intestinal inflammation (La Reau & Suen, 2018).

3.4. Free chitosan is the mode of presentation with the most significant impact on the composition and viability of the defined gut microbiota ecosystem

Given the limited information provided by individual gut isolate results, the impact of chitosan on a complex community was carried out by culturing a defined bacterial gut ecosystem within a chemostat. The defined gut microbiota community was exposed to the different forms of presentation of chitosan at two concentration levels (0.25 and 1 mg/mL). Compositional analysis results after 16S rRNA gene sequencing (Table 2, Figure 4 and Figure S3, supplementary information) showed differences in microbial diversity and differences in microbial composition. Table 2 shows the significant changes in diversity in the community profile for the chitosan-treated samples assessed by alpha-diversity analysis ($p<0.01$). Exposure to **F_CH**, especially the highest concentration (1 mg/mL) significantly reduced the community richness and microbial diversity in accordance to Chao index and Shannon index, respectively. This implies a significant alteration of the microbial community both in richness and diversity, which has been previously found in other studies with chitosan derivatives (Liu et al., 2020; Shang et al., 2017) and is in accordance with the results of bacterial isolates. In contrast, ecosystem diversity was slightly affected by the immobilized chitosan, being **AS5_CH** the support that produced the most pronounced changes on microbial diversity. Similar to the results with the individual isolates, exposure to solvent (acetic acid) or non-modified carriers had only a slight effect on the ecosystem composition according to the alpha-diversity results (see Table S9, supplementary information, for details).

Table 2. Index of richness and diversity relative for control samples (CT), and samples treated with 0.25 and 1 mg/mL of free chitosan (F) and chitosan immobilized in mesoporous silica (IMS), 5 µm-amorphous silica (IAS5), 10 µm-amorphous silica (IAS10) or cellulose (ICL) supports.

Group	ACE	Chao1	Simpson	Shannon	Fisher
CT	42	42	0.764	2.127	4.531
F_0.25	32	32	0.764	1.938	3.386
F_1	24	24	0.276	0.712	2.492
IMS_0.25	38	40	0.732	1.940	3.967
IMS_1	43	43	0.669	1.897	4.456
IAS5_0.25	40	40	0.638	1.604	3.912
IAS5_1	43	41	0.668	1.783	4.329
IAS10_0.25	39	39	0.729	2.002	4.157
IAS10_1	45	45	0.733	2.087	4.696
ICL_0.25	39	39	0.632	1.718	4.073
ICL_1	45	45	0.798	2.223	4.741

Chitosan treatment produced significant changes in the microbial community structure according to the heatmap of the relative abundance of the genera present in the defined community for the non-treated samples and the samples subjected to chitosan treatment (Fig. 4A). The abundance of the phylum Bacteroidetes, which was dominant with genus *Bacteroides*, showed a trend of increase after chitosan treatment, however, it was not significantly different from that in the control (Fig. S3, supplementary information). Otherwise, the genera from the phyla Firmicutes and Proteobacteria were significantly decreased. Therefore, the ratio of Firmicutes to Bacteroidetes was significantly reduced after treatment with chitosan in a similar way to the results seen in other studies with chitosan derivatives (Liu et al., 2020). The predominant families that were sensitive to chitosan were *Eubacteriaceae, Atopobiaceae, Acidaminococcacea* and

Enterococcaceae. In contrast, an increase in the abundance of the genera belonging to the families *Enterobacteriaceae, Veillonellaceae* and *Bacteroidaceae* was found after chitosan treatment (Fig. S3, supplementary information).

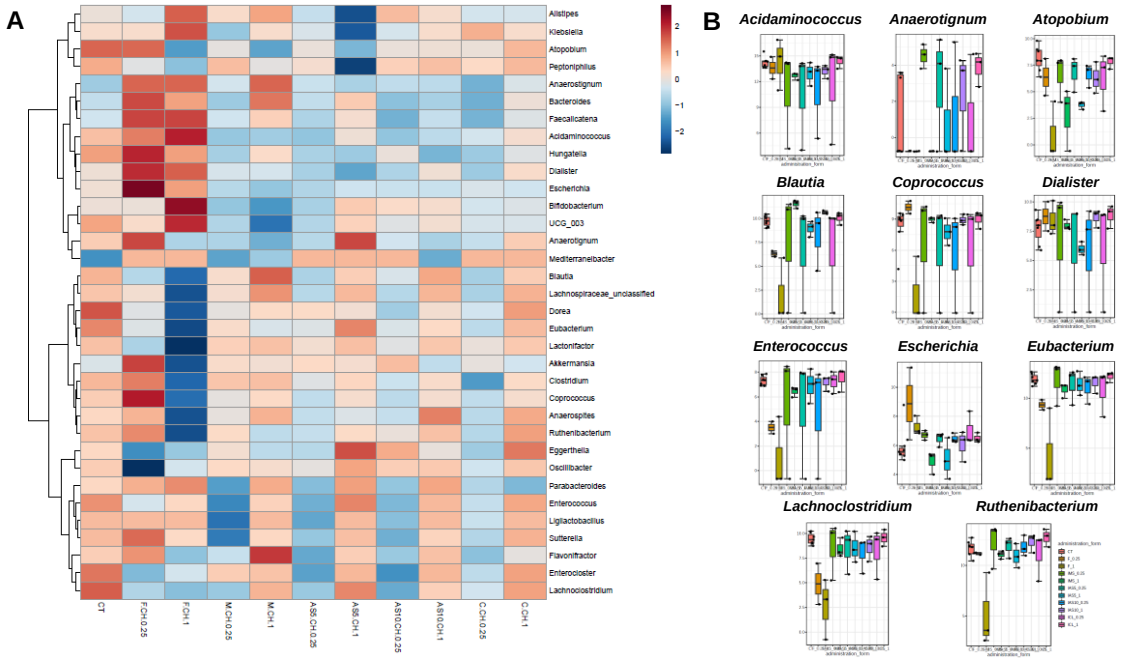


Figure 4. Microbiota composition in chemostat untreated samples or treated with 0.25 and 1 mg/mL of chitosan. (A) Heatmap showing the abundance of genera affected by chitosan treatment in microbiota samples. (B) Log-transformed count of genera significantly different (*p<0.01*) after exposure to chitosan. 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.

Focusing on relevant genera, the comparison of the relative abundances of the genera significantly affected by chitosan treatment according to univariate-statistical analyses (ANOVA/T-test) are presented in Fig. 4B. A low number of genera presented significant differences (*p<0.01*) in abundance, highlighting the reduction of the genera

Lachnoclostridium, *Anaerotignum*, *Blautia, Enterococcus, Eubacterium* and *Ruthenibacterium* in the samples treated with **F_CH**. The genera *Lachnoclostridium*, *Anaerotignum* and *Blautia* are members of the core gut microbiota belonging to the *Lachnospiraceae* family within the *Clostridium* cluster XIVa. An increase in the abundance of *Lachnospiraceae* is associated with different diseases, although the taxa of this family have repeatedly shown their ability to produce beneficial metabolites for the host. The *Blautia* genus is associated with a healthy state because some of its representative species are among the main SCFA producers of the gut microbiome (Vacca et al., 2020). The *Eubacterium* genus was mainly represented by a strain of *E. callanderi* a member of the core genotype of the genus *Eubacterium sensu stricto;* it is considered a beneficial species to human health as it is a butyrate producer (Mukherjee, Lordan, Ross, & Cotter, 2020). Therefore, imbalances in ecosystem composition due to diminished representation of key genera such as *Blautia* or *Eubacterium,* among others, have been linked with various human disease states (Mukherjee et al., 2020; van Zanten et al., 2014). These results are in accordance with the results obtained here that showed the significant impact of chitosan, mainly in its free form, on key species related to the production of SCFAs.

In contrast, the use of free or immobilized chitosan resulted mainly in an increase in the abundance of members of the genera *Escherichia* and *Dialister*, and to a lower extent *Bacteroides*, *Parabacteroides* and *Bifidobacterium*. *Escherichia* (family *Enterobacteriaceae*) and *Dialister* (family *Veillonellaceae*) are commonly associated with intestinal disease (Baldelli, Scaldaferri, Putignani, & Del Chierico, 2021; Han et al., 2021), but many members of these families are commensal species (Tenaillon, Skurnik, Picard, & Denamur, 2010). Conversely, *Bacteroides* and *Parabacteroides* (phylum Bacteroidetes) are considered dominant bacteria within the human intestinal tract with beneficial effects on the host (Lv et al., 2017). Similarly, *Bifidobacterium* (phylum Actinobacteria) is a genus considered as beneficial, with strains of several species

having been demonstrated to improve gut barrier function, stimulate the host immune system, activate provitamins, and modulate lipid metabolism (Hidalgo et al., 2012).

Similar results have been reported previously after exposing human gut microbiota to different non-digestible polymers. The use of chitosan as dietary adjuvant in humans was evaluated in the study of (Mrázek, Koppová, Kopečný, Šimůnek, & Fliegerová, 2010) resulting in an increase in the level of fecal *Bacteroides* in response to chitosan intake. Similarly, the supplementation of the diet of rats with COS–resistant starch complexes promoted the growth of saccharolytic bacteria such as *Bacteroides*, indicating a stronger fermentation of carbohydrates (Shang et al., 2017). This increase has been correlated with additional carbohydrate-degrading enzymes encoded by members of the Bacteroidetes, compared to Firmicutes, that support growth enhancement of these microorganisms (Yan et al., 2020).

The relative abundances of Bacteriodetes and Firmicutes were also altered in response to another antimicrobial polymer in mice fed ε-polylysine or ε-polylysine–pectin complexes (You et al., 2017). Neither ε-polylysine nor pectin are absorbed in the upper part of the intestine, therefore increasing the chance of their interactions with colonic microbiota. Exposure to ε-polylysine resulted in a transient perturbation of the ecosystem with an increase in Bacteriodetes and a concomitant decrease in Firmicutes that recovered to baseline values after final intake. However, the use of ε-polylysine–pectin complexes mitigated the antimicrobial effect of ε-polylysine. In the same way as for the immobilization of chitosan in this work, ε-polylysine–pectin complexes are used in food systems to stabilize the physicochemical properties of the polymer while preserving the antimicrobial properties. The protective interaction of the complexes described by (You et al., 2017) after exposure to the gut microbiota is similar to the results obtained in our study in which the different forms of immobilized chitosan resulted in lower impact on the defined gut microbiota ecosystem.

3.5. Free chitosan is the mode of presentation with the most significant impact on the metabolic behavior of the defined gut microbiota ecosystem

Besides determination of composition and viability of the defined ecosystem, the metabolic behavior of the community was evaluated after exposure to chitosan. Metabonomic profiles with more than 80 compounds were obtained from ^{1}H NMR analysis. Figure 5 shows the Principal Components Analysis (PCA) results of the control samples and the samples treated with two concentration levels of chitosan 0.25 and 1 mg/mL). The overall metabonome was significantly affected by chitosan and influenced by the mode of presentation and concentration. Bacterial metabolic responses were different for the samples treated with free or immobilized chitosan as can be observed in the bimodal clustering of samples: a group with the samples treated with **F_CH** and a second group containing the rest of the samples (control samples and samples treated with CH-coated supports). These results also agree with the effect showed by **F_CH** against the bacterial isolates or the bacterial ecosystem in the previous sections.

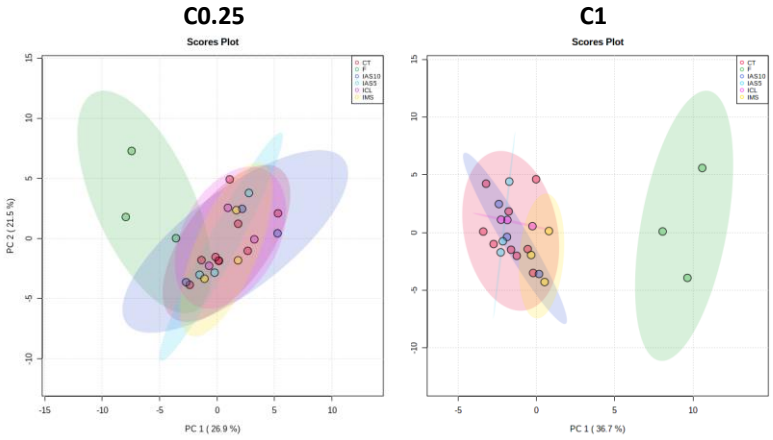


Figure 5. Principal Component Analysis (PCA) of metabonomic data after exposure to 0.25 (C0.25) and 1 (C1) mg/mL of chitosan. Coloring is used to distinguish the experimental groups, as indicated. CT: control samples, F: samples treated with free chitosan, IAS10: samples treated with chitosan immobilized on 10 µm-amorphous silica, IAS5: samples treated with chitosan immobilized on 5 µm-amorphous silica, ICL: samples treated with chitosan immobilized on cellulose, IMS: samples treated with chitosan immobilized on mesoporous silica.

The metabolite concentrations that were significantly different between the control group and the different modes of administration of chitosan are shown in Figure 6. The use of **F_CH** resulted in significant concentration changes on fermentation by-products. Exposure to **F_CH** resulted in an increase of the concentration of some amino acids, such as tyrosine and valine, with a reduction of alanine (Fig. 6A). In relation to SCFAs (Fig. 6B), acetate, propionate and butyrate were the most prevalent SCFAs, although concentrations of these compounds were similar for all the samples. The production of overall SCFAs was reduced after treatment with **F_CH** (data not shown), except for formate, but these changes were not statistically significant ($p>0.05$). Similarly, total SCFAs concentration for immobilized chitosan samples was slightly higher than for **F_CH** or control groups. The most pronounced changes resulted in the production of the SCFA valerate and the BCFA isobutyrate; the production of both of these was significantly reduced ($p<0.05$) by the treatment with **F_CH**. The reduction of SCFAs production can be related to the decrease of key SCFA-producing species belonging to the Firmicutes phylum, as inferred from our results with both single isolates and complex ecosystems.

Lipid metabolism (Fig. 6D) was also affected by **F_CH** with an increase of choline (a derivative of phospholipid breakdown) and a decrease of glycocholic acid (secondary bile acid) compared to the other conditions. Ethanol and methanol concentrations (Fig. 6E) increased after exposure to **F_CH**. Significant differences can also be found in other metabolites, such as carboxylic acids (Fig. 6C) and phenols (Fig. 6F) after exposure to **F_CH**. The significant changes observed in the concentration of various metabolites derived from macronutrient metabolism endorse the results obtained in the compositional analysis (section 3.4), indicating a partial inhibition of the microbial population of the ecosystem. In contrast, the exposure to the CH-coated supports generally resulted in non-significant changes compared to the control group.

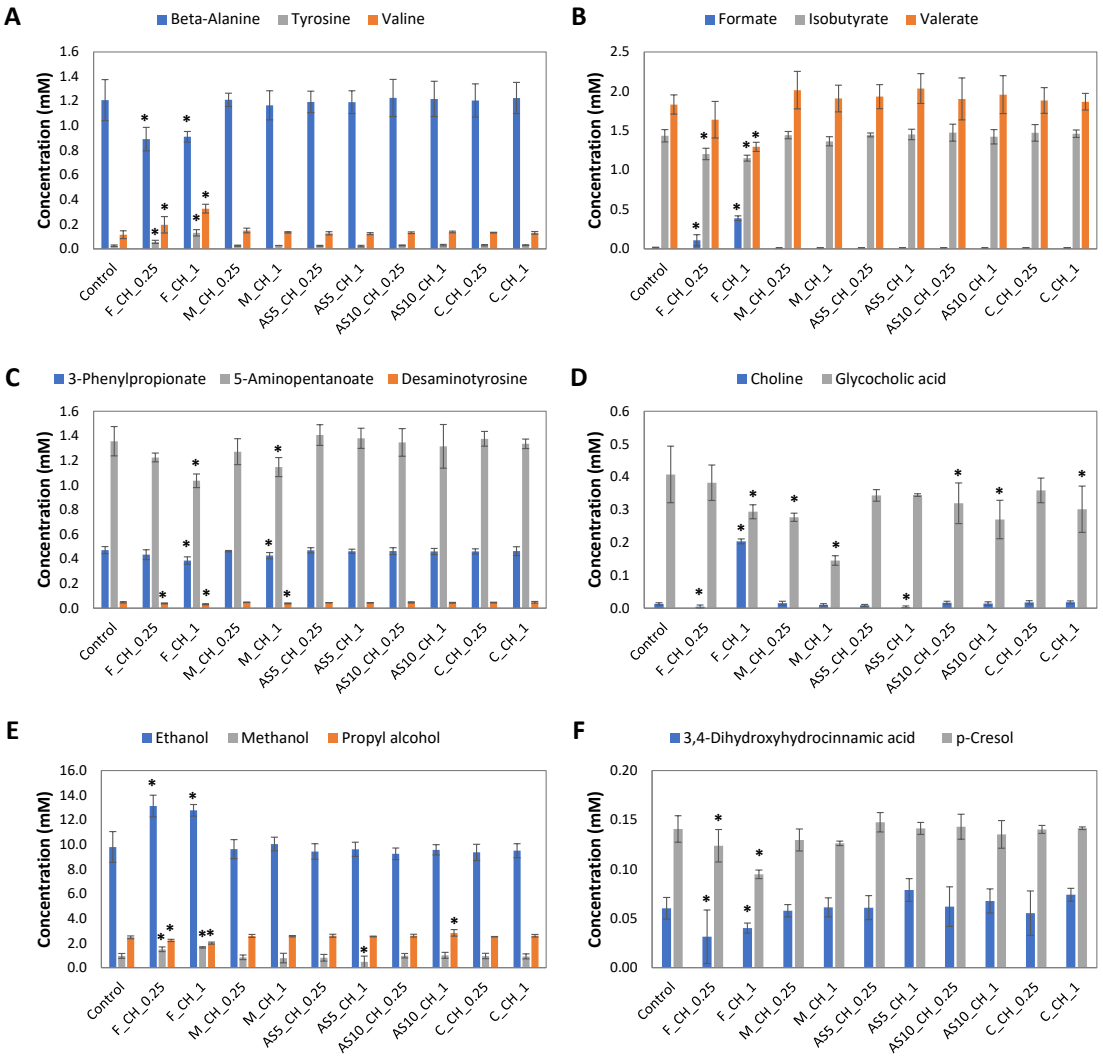


Figure 6. Concentration (mM) of metabolites significantly affected (*p<0.05*) by exposure to 0.25 mg/mL or 1 mg/mL of chitosan in its free form (F) or immobilized in mesoporous silica particles (M), 5 µm-amorphous silica particles (AS5), 10 µm-amorphous silica particles (AS10) and cellulose particles (C), in comparison to control samples. Metabolites are grouped by category: (A) amino acids, (B) SCFAs and BCFAs, (C) carboxylic acids, (D) lipid-related metabolites, (E) alcohols, and (F) phenolic compounds.

4. Conclusions

In this study, the influence of the antimicrobial biopolymer chitosan administered in different modes of presentation (free or immobilized) on the gut microbiota was evaluated in two stages. As a first step, the impact of chitosan on representative single gut microbes was assessed, with free chitosan demonstrating a greater antimicrobial effect. As well,

we found the susceptibility of different strains and species related to the tested isolates was significantly diverse after chitosan exposure. Therefore, considering the dissimilarities between species of a related genus and the inter-individual differences on the effect of chitosan on gut microbes, studies with a large number of species should be done to extrapolate the results. Accordingly, as a second stage, a more complex *in vitro* system based on a defined gut microbiota ecosystem was used to evaluate the influence of chitosan on the compositional stability and metabolic behavior of this model gut microbiota. The composition and viability of the defined community was altered by chitosan mainly in its free form. The disturbance of the ecosystem structure was mitigated when chitosan was immobilized in the different carriers.

The results obtained from study of the individual isolates and from the defined ecosystem are in accordance in terms of the potential of inhibitory properties of free and immobilized chitosan, but differ in terms of the susceptibility of the relevant genera when alone or as part of a community. Thus, it is necessary to perform studies that simulate a real environment, such as the chemostat systems and *in vivo* studies, to obtain results that can be generalized. The results of this study provide valuable information about the biocompatibility of free and immobilized chitosan as potential food additives, confirming the importance of evaluating the impact of new ingredients and materials included in food on the gut microbiota that is indispensable for human health.

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary information to this article can be found online.

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