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Potential of High- and Low-Acetylated Galactoglucomannooligosaccharides as Modulators of the Microbiota Composition and Their Activity

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Potential of High- and Low-Acetylated Galactoglucomannooligosaccharides as Modulators of the Microbiota Composition and Their Activity: A Comparison Using the *In Vitro* Model of the Human Colon TIM-2

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ABSTRACT: High- and low-acetylated galactoglucomannooligosaccharides (GGMOS_Ac and GGMOS, respectively) were assayed as substrates in the TIM-2 *in vitro* colon model using, as inoculum, fecal microbiota from the elderly. The effects on the microbiota and their activity were also compared to a standard ileal efflux medium (SIEM). GGMOS resulted in higher organic acid productions and higher short-chain fatty acids/total organic acid molar ratios. Although comparable Actinobacteria abundances were observed with both substrates, GGMOS fermentation led to higher Firmicutes/Bacteroidetes ratios and lower Proteobacteria percentages than GGMOS_Ac. No differences were found concerning the percentages of beneficial genus such as *Blautia*, *Faecalibacterium*, *Coprococcus*, or *Bifidobacterium*. However, higher bacterial diversities and numbers of genera such as *Oscillospira* and *Lachnospira* were found with GGMOS_Ac. This suggests that GGMOS would be more suitable substrates for the elderly, even though GGMOS_Ac promoted positive effects that support the interest of further research using these oligosaccharides as "carriers" of desired substituents.

KEYWORDS: *galactoglucomannooligosaccaharides, GGMOS, Pinus pinaster, acetylated, SIEM, prebiotics, gut microbiota, elderly, TIM-2*

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INTRODUCTION

The colonic microbiota is a complex ecosystem consisting of trillions of microorganisms, mainly bacteria. The existence of an unbalanced microbiota (dysbiosis) has been related to a wide range of pathologies, including inflammatory bowel disease (IBD), obesity, celiac disease, cancer, diabetes, and even neurological disorders.[1]

Although the gut microbiota has shown to be long-term stable within individuals,[2] there are several factors, such as aging, that can adversely alter the balance among microbial communities and their metabolism, affecting the health of the elderly. In this context, "healthy aging" has become a real issue for national health systems given the intrinsic health detriment associated with the elderly population.[3]

When comparing the gut microbiota of young adults and elderly people, changes in the dominant species, a decrease in bacteria diversity, and abundance as well as lower presence of short-chain fatty acids (SCFAs) were observed.[4] In particular, the elderly have shown reduced levels of Firmicutes (mainly *Clostridium* cluster XIVa and *Faecalibacterium* spp.) and Actinobacteria (including *Bifidobacterium* spp.), lower Firmicutes/Bacteroidetes ratios, and a diminished presence of bacterial species such as *Anaerostipes hadrus* and *Eubacterium hallii*. In addition, microbiota from elderly individuals have shown an increased presence of Proteobacteria and Bacteroidetes phyla, methanogens, sulfate-reducing bacteria, and also greater contents in bacteria belonging to the genus *Bacteroides* spp. and *Clostridium* spp.[5−7] Furthermore, variations in the total SCFA concentration and their ratio have been observed, which have been associated with a change from microbial saccharolytic metabolism toward a predominantly putrefactive one, which leads to the production of several toxic metabolites.[8,9] Some of these observations were also found when comparing healthy people and IBD patients.

To counteract the adverse effects of an unbalanced microbiota, dietary interventions have been suggested as a suitable approach and several scientists have focused their research on the elderly population due to their particular vulnerability.[10,11] The use of prebiotics has been suggested as a promising strategy, including the study of possible associations between prebiotic substrates and specific shifts in microbial communities and/or their metabolism, results of which will provide valuable information to the prebiotic field.[12]

In this context, dynamic *in vitro* models constitute a relevant and reliable tool for assessing the effects promoted by the food components. Among these type of models, the TNO's dynamic *in vitro* model of the proximal colon (nicknamed TIM-2) has been widely used to study the effects of diverse carbohydrates in the gut microbiota, including maize-based carbohydrates,[13] galacto-oligosaccharides,[14] long-chain arabinoxylans,[15] agave fructan products,[16] arabinogalactan,[17] mango peel,[18] inu-

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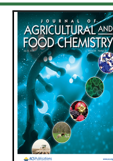

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lin,[15−17] linear short-chain fructooligosaccharide (FOS),[16] or byproducts from orange bagasse and passion fruit peels.[19]

Galactoglucomannooligosaccharides (GGMOSs) are mannan-derived oligosaccharides that can be manufactured from biomass sources such as *Pinus pinaster* wood. These oligosaccharides have previously shown bifidogenic potential on *in vitro* experiments;[20] however, their prebiotic potential has not yet been deeply evaluated.

The aim of this work was to compare the effects of high- and low-acetylated galactoglucomannooligosaccharides (GGMOS-Ac and GGMOS, respectively) on the composition and the activity of the elderly gut microbiota, using the TIM-2 dynamic *in vitro* model of the proximal colon, to assess the potential of both substrates as strategies to prevent and/or treat the dysbiosis caused by aging. GGMOS_Ac were included in this work for evaluating their potential for modifying the gut microbiota and for increasing the total SCFA (by carrying acetyl groups than can be converted into acetic acid) with potentially reduced gas generation. This work is a new contribution to the knowledge of the prebiotic field focused on elderly gut microbiota, as it is the first time that high-acetylated GGMOS, obtained by chemical derivation, are proposed as prebiotic candidates and compared to low-acetylated GGMOS by *in vitro* fermentation in this population.

MATERIALS AND METHODS

Substrates. *Galactoglucomannooligosaccharides (GGMOS).* Oligosaccharides derived from galactoglucomannan were obtained from *P. pinaster* wood chips following the two-step hydrothermal processing described by Rivas et al.[20] with minor modifications related to the scale-up from the laboratory reactor (V = 3.75 L) to a mini-pilot plant scale (18.75 L) using the same severity factor.[20,21] To increase the purity of the GGMOS, the resulting liquors were subjected to membrane filtration using a regenerated-cellulose spiral wound membrane of 1 kDa cutoff, according to the scheme described by Rivas et al.[20] The liquid phase, which contained the purified GGMOS, was then freeze-dried and stored until use.

Acetylated Galactoglucomannooligosaccharides (GGMOS_Ac). A sample of the freeze-dried GGMOS was submitted to an acetylation process following the method proposed by Thielemans and Wool[22] for the esterification of lignin with some modifications. Briefly, freeze-dried GGMOS were suspended in acetic anhydride in a 2:1 (g acetic anhydride/g GGMOS) ratio and heated up to 50 °C in a water bath. Then, 0.01 mL of 1-methylimidazole catalyst per gram of GGMOS was added to the medium and the suspension was allowed to react for 40 h to assure the total acetylation of the hydroxyl groups. The resulting solid was washed with distilled water to remove the catalyst and freeze-dried.

Standard Ileal Efflux Medium (SIEM). SIEM was included in the experimental design as a control. This medium is composed mainly of starch, pectin, xylan, arabinogalactan, amylopectin, protein, vitamins, salts, Tween 80, and ox bile,[13] and it does not require predigestion.[13,16]

GGMOS and GGMOS_Ac Chemical Characterization. *High-Performance Liquid Chromatography (HPLC).* The content in oligomers as well as in acetyl groups was determined in GGMOS and GGMOS_Ac by difference of the respective monosaccharides and acetic acid concentrations[23] in the samples measured directly by HPLC (Agilent 1260 series, Agilent Technologies, Santa Clara, CA) and after quantitative acid hydrolysis (QAH), which was performed following the procedure NREL/TP-510-42618,[24] with some modifications: 2 mL of 72% H_2SO_4 was added to 0.20 g of the oligosaccharides and incubated in a water bath at 30 °C for 30 min and then treated at 121 °C in an autoclave for 30 min at a H_2SO_4 concentration of 4%. The concentrations of mannose, glucose, xylose, galactose, and arabinose oligomers were then determined in QAH samples by HPLC using an Aminex HPX-87P column (BioRad, Hercules, CA) operating at 80 °C using distilled water as the mobile phase (flow rate: 0.4 mL/min). The content in acetic acid was analyzed by HPLC using an Aminex HPX-87H column (BioRad, Hercules, CA) working at 60 °C and using 0.003 M H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. A refractive index (RI) detector thermostated at 35 °C was used in these analyses.

High-Pressure Size-Exclusion Chromatography (HPSEC) Analysis of Substrates. HPSEC analysis was carried out to determine the molar mass distribution of the purified GGMOS according to Rivas et al.[20] with some modifications. Two TSKGel G3000PWXL columns (Tosoh Bioscience, Stuttgart, Germany) coupled in series were used in an Agilent 1200 HPLC equipped with an RI detector. The columns were kept at 70 °C, and Milli-Q water was used as the mobile phase at a 0.5 mL/min flow rate. Aqueous solutions of dextrans with different molecular weights (1000−80 000 g/mol) were used as standards for calibration. Agilent gel permeation chromatography (GPC) data analysis software was employed to calculate the number average molecular weight (M_n), the weight average molecular weight (M_w), and the polydispersity index (D).

Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectra of substrates were also obtained using a Thermo Nicolet 6700 instrument (Thermo Scientific, Germany) equipped with an attenuated total reflection (ATR) diamond accessory. Spectra were recorded from 4000 to 400 cm^{-1} with 34 scans and a resolution of 4 cm^{-1}.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Analysis. GGMOS samples were analyzed by MALDI-TOF MS using an Autoflex III smartbeam MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) operating in the reflector positive ion mode using a smartbeam nitrogen laser (337 nm) with an accelerating potential of 20 kV and a frequency of 200 Hz. Mass spectra were produced rastering 200 laser shots in the range m/z 500−3500. External mass calibration was performed using calibration standards (Bruker Daltonik, Germany) for the range m/z 700−4000.

Sample preparation involved a dried-droplet methodology where 2 μL of 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 mg/mL in 50% ACN/0.1% trifluoroacetic acid (TFA) v/v) was directly applied on an MTP AnchorChip 800/384 TF MALDI target (Bruker Daltonik, Bremen Germany). Subsequently and before drying the matrix solution, 2 μL of the sample (1 mg/mL in water with 0.1% TFA) was added and allowed to dry at room temperature.

***In Vitro* Fermentation in the TNO's Proximal Colon Model (TIM-2).** *Collection, Preparation, and Standardization of Fecal Samples.* Stool samples were obtained from healthy elderly donors recruited in Spain (n = 6, three males and three females, average age 75 ± 8 years), who followed a normal omnivorous diet and had not consumed antibiotics, laxatives, or pre- or probiotics during at least 2 months before sample donation. All of them signed an informed consent prior to fecal donation. The collection was performed at home by the donors in airtight plastic boxes containing an anaerobic environment generation bag (AnaeroGen, Oxoid, Cambridge, U.K.). Within 2 h after collection, the fresh samples were transferred to an anaerobic chamber (Bactron II, Shel-lab). Tubes containing the fecal material from each donor were prepared inside the anaerobic cabinet following the procedure recommended as optimal for *in vitro* studies by Aguirre et al.[25] Briefly, each sample was weighed and mixed in a proportion of 1:1 (w/w) with a dialysate preparation containing NaCl, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, cysteine hydrochloride, ox bile, and $FeSO_4 \cdot 7H_2O$ (4.5, 2.5, 0.5, 0.45, 0.4, 0.05, and 0.005 g/L, respectively), and 15% (final concentration) glycerol was added to the homogenate. Fecal slurries were individually homogenized, filtered through a sieve to remove large undigested particles, and split in sterile 50 mL tubes (up to 35−45 mL of volume). To guarantee cell viability, tubes were snap-frozen with liquid N_2 (−196 °C) and kept at −80 °C until use. Before initiating the *in vitro* assays, a sample from each donor was mixed to prepare a standardized pool as described by Aguirre et al.,[25] which was shown to be a suitable technique for fermentation experiments performed using the TIM-2 system[26] and allows the use of the same inocula in all of

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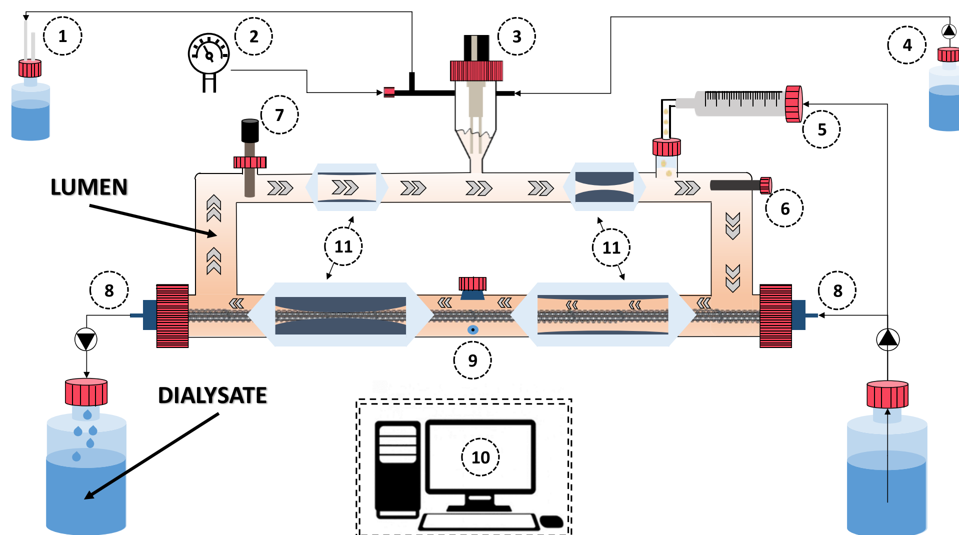

Figure 1. Schematic representation of the TIM-2 dynamic *in vitro* model of the proximal colon: (1) N_2 gas outlet, (2) N_2 gas inlet, (3) level sensor, (4) NaOH pump, (5) feeding syringe, (6) temperature sensor, (7) pH electrode, (8) dialysate system (including the hollow fiber membranes inside the TIM-2 lumen for metabolite permeation), (9) sampling port, (10) computer control system, and (11) peristaltic compartments covered by temperature-controlled water jackets.

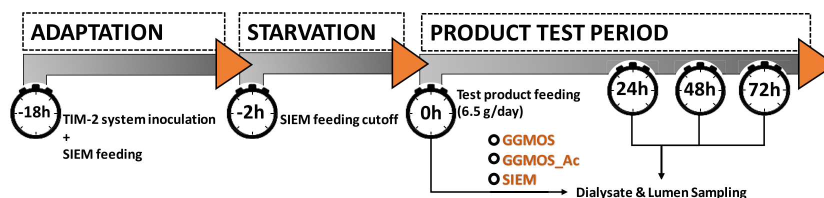

Figure 2. TIM-2 experimental timeline performed for the assessment of the prebiotic potential of three different substrates in the gut microbiota from elderly donors. GGMOS, galactoglucomannooligosaccharides; GGMOS_Ac, acetylated galactoglucomannooligosaccharides; SIEM, standard ileal efflux medium.

the assays that took place over several weeks, with four experiments/week. In short, tubes containing the individual microbiota were introduced in a water bath at 37 °C for 1 h and then transferred to an anaerobic cabinet (Bactron IV, Sheldon manufacturing, Cornelius, OR), pooled, and mixed with prereduced dialysate.

TIM-2 System Experimental Setup. The TIM-2 *in vitro* dynamic model is a validated system that allows the simulation of the anaerobic environment and conditions in the proximal colon such as the peristaltic movements and the absorption of the generated microbial metabolites.[27] Figure 1 shows a schematic representation of a TIM-2 working unit. A detailed description of this model can be found in previous works.[13,27,28]

The workflow process of the experiments performed in this work is summarized in Figure 2. Prior to microbiota inoculation, 120 mL of prereduced dialysate solution (see above) with vitamins (1 mg/L menadione, 2 mg/L D-biotin, 0.5 mg/L cobalamin, 10 mg/L pantothenate, 5 mg/L nicotinamide, 5 mg/L *p*-aminobenzoic acid, and 4 mg/L thiamine, all purchased from Sigma-Aldrich) was introduced in each TIM-2 unit and N_2 was allowed to flow inside the system for a minimum of 3 h. Then, 55 mL of the fecal pool was inoculated in each unit through the sample port using a syringe. Subsequently, the feeding system containing SIEM was connected, letting this carbohydrate source flow inside the system at a rate of 2.5 mL/h (adaptation period) during 16 h, allowing the gut microbiota to adapt to the system conditions. Later, the SIEM feeding was stopped and the microbiota was left without a new carbohydrate source during 2 h (starvation period), the time necessary to consume the residual SIEM.[28] Before introducing the substrates, a sample of the lumen was taken at t = 0 h for basal microbiota (BMB) characterization. For testing the substrates, the carbohydrates of SIEM were replaced by the GGMOS or GGMOS_Ac, leaving all other components in the medium constant. Thereafter, the pH of the medium was adjusted to 5.8, split in the daily dosages, and frozen until use. Before being introduced in the artificial colon, dosages were thawed in a water bath at 37 °C for 1 h. The total carbohydrate addition in each experiment was 19.5 g of the target oligosaccharides (or polysaccharides in the case of SIEM), being administered at a constant rate of 6.5 g/day during the 3 days at an approximate rate of 2.5 mL/h. Samples were taken from the lumen and dialysate (which contains the compounds permeated through the membranes located inside the system simulating the intestinal absorption; see Figure 1) at t = 24, 48, and 72 h. In addition, at 24 and 48 h, an additional volume resulting in a total volume of 25 mL of lumen content (samples + additional volume) was withdrawn and replaced by dialysate to mimic the material movement from the proximal to distal colon, leaving also space for the microbiota to grow. All of the fermentations were carried out in duplicate.

Microbiota and Metabolite Production Analyses. The analyses of microbiota and metabolite production were performed according to the methods described elsewhere.[29,30]

For microbiota analyses, first, the DNA of the lumen samples was isolated using standard molecular biology kits from ZYMO Research provided by BaseClear (Leiden, The Netherlands). A two-step polymerase chain reaction (PCR) amplification was employed to amplify the 16S rRNA gene (V3 and V4 regions), with barcoding and library preparation according to Illumina (Eindhoven, the Netherlands). The Illumina MiSeq system was employed for sequencing, and the 1.8.3 of BCL2FASTQ pipeline version was used to convert sequences into FASTQ files, using a quality cut based on the quality level of Phred (Phred quality score). Microbial compositional analyses were performed using the software package of Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.0), and sequences were

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classified employing GreenGenes (version 13.8) as the rRNA gene database ref 31.

The organic acids generated during the fermentation were analyzed by ion-exclusion chromatography by the company Brightlabs (Venlo, The Netherlands). Briefly, 10 μL of lumen or dialysate samples (previously centrifuged and diluted in 1.5 mM H_2SO_4) was injected in an 883 chromatograph (IC, Metrohm, Switzerland) equipped with a Transgenomic ICSep ICE-ION-300 column and a MetroSep RP2 Guard column (T = 65 °C; flow rate = 0.4 mL/min). A conductivity detector working in the positive mode was employed for analyte detection.

Statistical Analyses. The software package R (3.6.2) (R Core Team, 2013) was used to perform the following statistical analyses: on the one hand, for those variables fulfilling the assumptions of normality and homogeneity of variances, one-way analysis of variance (ANOVA), followed by Duncan's *post hoc* tests in the case of significance, was performed to assess the existence of differences among groups (considered significant at $p < 0.05$). On the other hand, R was employed to carry out the Kruskal−Wallis nonparametric tests and Dunn's *post hoc* tests for the assessment of significant differences across substrate groups at phylum and genus levels and to determine the existence of correlations between the relative abundance of operational taxonomic units (OTUs) and metabolites using Spearman correlation tests. Multiple comparisons were corrected using the false discovery rate (FDR), and q-values were considered significantly different when <0.05. A linear discriminant analysis (LDA) effect size (LEfSe)[32] was performed to find discriminant OTUs responsible for the main differences among interventions using the relative abundances from the OTUs' tables generated in QIIME using the software package Galaxy (online: https://huttenhower.sph.harvard.edu/galaxy). Principal coordinate analyses (PCoA; weighted and unweighted UniFrac) were made in QIIME.

Random Forest analyses were performed from within METAGENassist (http://www.metagenassist.ca/METAGENassist/faces/Home.jsp), using generation of 500 trees, with 10 predictors to try for each node.

RESULTS AND DISCUSSION

Substrate Chemical and Structural Characterization. The data for the composition of the purified GGMOS and GGMOS_Ac is summarized in Table 1. As it can be observed,

Table 1. Chemical Compositions of GGMOS and GGMOS_Ac Fractions[a]

component	GGMOS content (g/g substrate)	GGMOS_Ac content (g/g substrate)
glucan	0.160 ± 0.005	0.100 ± 0.001
mannan	0.615 ± 0.020	0.359 ± 0.003
galactan	0.043 ± 0.001	0.034 ± 0.001
xylan	0.041 ± 0.001	0.038 ± 0.001
arabinan	0.002 ± 0.001	0.001 ± 0.001
AcO	0.059 ± 0.005	0.429 ± 0.008
ONVC	0.081 ± 0.032	0.040 ± 0.013
molar ratio AcO/HX	0.27	3.28

[a]Average ± standard deviation (SD) (n = 3). AcO, acetyl groups; HX, hexoses (glucan + mannan + galactan); ONVC, other nonvolatile compounds.

high-purity substrates (92% for GGMOS and 96% for GGMOS_Ac) were obtained following the proposed scaled-up scheme. These purity values are in agreement (or were even better) with the ones found in several commercial prebiotics. Table 1 also shows that the main difference between both substrates is their content in acetyl groups, the acetyl groups/hexoses molar ratio being 0.27 for GGMOS (approximately, one acetyl group/four hexose residues) and 3.28 for GGMOS_Ac (approximately, the maximum acetylation degree achievable). To attribute the effects observed to a specific substrate, a detailed chemical characterization is necessary. Therefore, various analytical techniques were used in this work.

The HPSEC analysis of GGMOS (Figure S1) allowed determining the molecular weight distribution, obtaining values of 826.8 g/mol, 2177.0 g/mol, and 2.6 for M_n, M_w, and D parameters, respectively. This corresponds to degrees of polymerization (DPs) of 5.1 and 13.4 using the M_n and M_w values, respectively. Despite these values being lower than others found in the bibliography for native oligosaccharides,[33] they are comparable to those obtained with treatments at increased severities.[34]

The FTIR spectrum obtained for GGMOS (Figure S2) was in agreement with a typical one for glucomannan polysaccharides,[35] with a strong absorption band at 3360 cm^{-1} corresponding to hydroxyl groups that disappear completely after acetylation, whereas the band corresponding to ester groups strongly increases (1737 cm^{-1}) due to the acetylation process. The total removal of the catalyst was confirmed by comparing its FTIR spectra and the one of GGMOS-Ac.

The degree of polymerization (DP) of the GGMOS was also analyzed by MALDI-TOF MS. Most of the oligomers found in the spectra (Figure S3 and Table S1) corresponded to adducts with sodium or potassium, with the main products being hexose chains with DPs in the range 3−16 (probably made up of mannose, glucose, and galactose as can be deduced from the data shown in Table 1). Moreover, this analysis suggests that the GGMOS are partially substituted with some pentoses (mainly xylose). The most intense signals were assigned to a series of partially acetylated oligomers with DP between 3 and 6. The highest signal was assigned to an oligomer made up of six hexoses containing two acetyl groups.

Changes in Microbial Composition upon Substrates Fermentation. For simplicity, the data considered in this section does not include those phyla or genera whose presence across samples was lower than 0.1%. Data referring to the different carbohydrate experiments corresponds to the average values from t = 24−72 h (n = 6 for each substrate), while the data corresponding to basal microbiota (t = 0 h, denoted BMB throughout the manuscript) includes the average of five independent experiments (one outlier was discarded). To test whether the administration of GGMOS, GGMOS_Ac, or SIEM promoted significant variations in gut microbial communities in comparison with the basal gut microbiota and to assess the existence of significant differences between the bacterial composition found in substrate groups, the (FDR-corrected) nonparametric Kruskal−Wallis tests followed by the *post hoc* Dunn test at both phylum and genus levels were applied to the experimental data.

Microbiota Modulation at the Phylum Level. The results obtained for the microbiota phyla relative abundances at t = 0 h and in each *in vitro* experiment (average of sampling points 24−72 h) are shown in Figure 3. Kruskal−Wallis nonparametric tests revealed that the type of substrate significantly affected the abundances of five out of the six main phyla (q-values <0.05) but not the phylum Bacteroidetes, whose relative abundance average varied in the range of 22.39−26.73%. The *post hoc* Dunn test showed that GGMOS and GGMOS_Ac did not show significant differences between them in Firmicutes, Actinobacteria, or Tenericutes abundances, while the presence of the phyla Proteobacteria and Euryarchaeota showed a

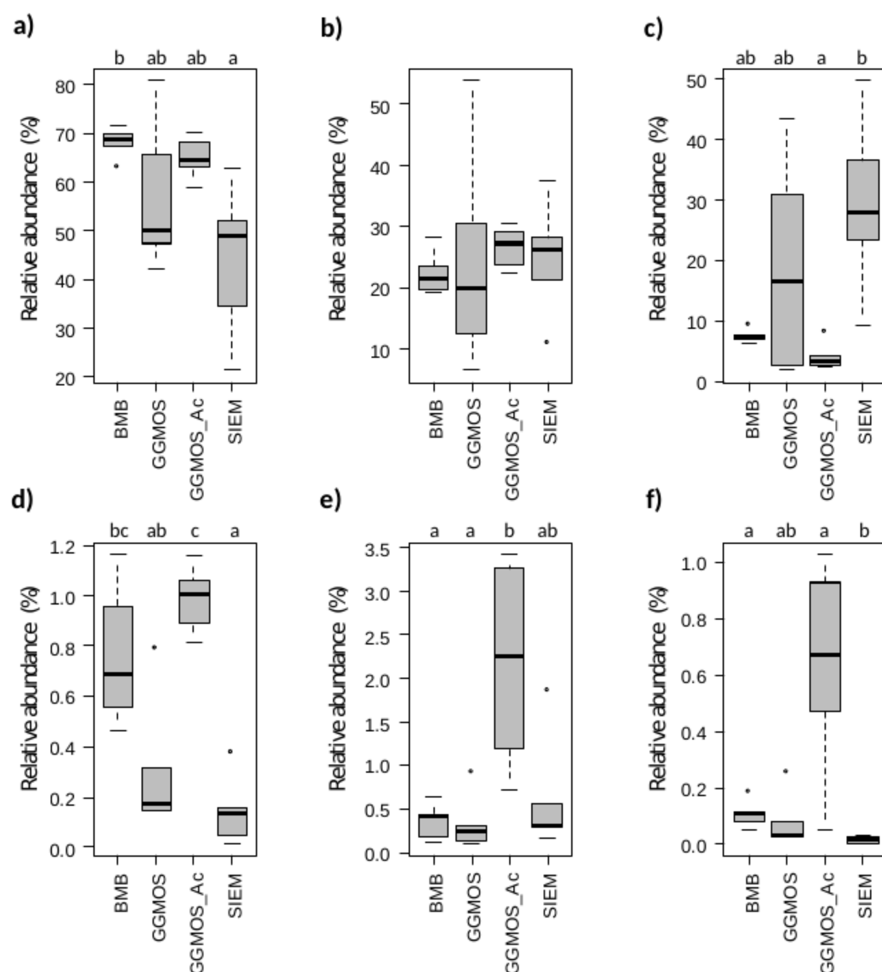

Figure 3. Relative abundance at the phylum level measured in samples obtained from the TIM-2 lumen: (a) Firmicutes, (b) Bacteroidetes, (c) Actinobacteria, (d) Euryarchaeota, (e) Proteobacteria, and (f) Tenericutes. Data for basal microbiota (BMB) corresponds to microbiota composition at $t = 0$ h ($n = 5$), while GGMOS, GGMOS_Ac, and SIEM box and whiskers include the values of the relative phylum abundances achieved at fermentation times of 24, 48, and 72 h ($n = 6$ for each substrate). Dunn's *post hoc* results are indicated with letters above those phyla that showed significant differences based on Kruskal–Wallis nonparametric tests. For Kruskal–Wallis tests, differences were considered significant when q-values (p values adjusted by the false discovery rate, FDR, for multiple comparisons) were <0.05. Groups sharing an identical letter are not significantly different according to Dunn's tests. GGMOS, galactoglucomannooligosaccharides; GGMOS_Ac, acetylated galactoglucomannooligosaccharides; SIEM, standard ileal efflux medium.

significant increase upon GGMOS_Ac fermentation with respect to GGMOS, which may be due to the lower carbohydrate/protein ratio, as will be discussed later. These results suggest that GGMOS would be a better substrate than GGMOS_Ac for their potential use as prebiotics in elderly people as previous research has shown an increment in Proteobacteria (which includes the *Enterobacteriaceae* family) in the elderly population.[36] Regarding the Actinobacteria, GGMOS allowed the maintenence of important levels of this phylum (comparable to those obtained with SIEM or GGMOS_Ac), which is very interesting for microbiota balance in elderly people, as it has been demonstrated that Actinobacteria start decreasing along the aging proccess.[37] On the other hand, the fermentation of the polysaccharides mixture in SIEM resulted in a significant reduction of the Firmicutes phylum in comparison with that observed in the basal microbiota. In addition, it is necessary to state that the Firmicutes/Bacteroidetes (F/B) ratio significantly increased after 72 h of GGMOS fermentation (from 3.12 at $t = 0$ h to 6.98 at 72 h, one-way ANOVA $p < 0.05$), whereas this ratio did not statistically differ with the measured one at $t = 0$ h in GGMOS_Ac or SIEM experiments (obtaining values of 2.35 and 2.11 after 72 h, respectively). This increment in the F/B ratio might be desirable for elderly microbiota modulation toward a profile more similar to young adults, as lower F/B ratios have been found in the elderly.[38] These changes in the microbiota composition could lead to changes in the ratio between the different major SCFAs, which could, as a consequence, affect the host health. For example, as has been reported, Bacteroidetes produce mainly propionate and acetate, while microorganisms belonging to Firmicutes produce primarily butyrate,[39] this last one being a target metabolite for the elderly.[7]

In global terms and taking into account the microbiota at the phylum level, GGMOS would appear, at first glance, the best substrate if the aim was to modulate the microbiota of elderly people, as it allows one to achieve comparable levels of Actinobacteria and Firmicutes to those obtained with GGMOS_Ac but higher F/B ratios and lower Proteobacteria abundances.

Bacterial Communities' Changes at the Genus Level. The methodology employed for microbiota characterization

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Table 2. Genus Relative Abundances before and after TIM-2 Fermentations[a]

genus	relative abundance (%)				q-value
	BMB	GGMOS	GGMOS_Ac	SIEM	
Faecalibacterium	2.89 ± 0.59[b]	1.21 ± 0.08[ab]	0.36 ± 0.34[a]	1.42 ± 0.26[b]	0.003
Coprococcus	1.23 ± 0.41[b]	0.15 ± 0.10[a]	0.55 ± 0.08[ab]	0.14 ± 0.17[a]	0.003
Methanobrevibacter	0.74 ± 0.27[a]	0.28 ± 0.23[ab]	0.94 ± 0.14[a]	0.11 ± 0.09[b]	0.003
Parabacteroides	0.70 ± 0.28[a]	0.18 ± 0.16[ab]	0.76 ± 0.23[a]	0.03 ± 0.01[b]	0.003
Sutterella	0.20 ± 0.13[ab]	0.04 ± 0.02[a]	0.50 ± 0.09[b]	0.08 ± 0.04[a]	0.003
f_Clostridiaceae.g_Other	1.64 ± 0.42[a]	0.42 ± 0.23[ab]	1.54 ± 0.41[a]	0.19 ± 0.18[b]	0.003
*f_Christensenellaceae.g_*	0.14 ± 0.11[bc]	0.02 ± 0.01[ab]	2.41 ± 1.27[c]	0.01 ± 0.01[a]	0.003
*o._RF39.f_.g_*	0.10 ± 0.05[ab]	0.07 ± 0.09[ab]	0.64 ± 0.37[a]	0.01 ± 0.01[b]	0.003
*f_Mogibacteriaceae.g_*	0.16 ± 0.09[ab]	0.03 ± 0.02[a]	0.32 ± 0.08[b]	0.02 ± 0.02[a]	0.003
*f_Rikenellaceae.g_*	0.11 ± 0.07[bc]	0.01 ± 0.00[a]	0.30 ± 0.07[b]	0.03 ± 0.02[ac]	0.003
Bifidobacterium	2.74 ± 0.51[ab]	1.29 ± 0.92[a]	1.41 ± 0.88[a]	11.21 ± 10.03[b]	0.007
Megasphaera	0.27 ± 0.15[a]	0.17 ± 0.22[a]	3.19 ± 1.13[b]	0.42 ± 0.43[ab]	0.007
Phascolarctobacterium	0.36 ± 0.04[ab]	0.32 ± 0.17[ab]	0.48 ± 0.10[a]	0.05 ± 0.04[b]	0.007
Lachnospira	0.20 ± 0.10[b]	0.02 ± 0.02[a]	0.31 ± 0.18[b]	0.06 ± 0.06[ab]	0.007
*o_Clostridiales.f_.g_*	5.93 ± 1.36[ab]	10.39 ± 4.25[a]	7.38 ± 0.95[a]	3.41 ± 2.02[b]	0.007
o_Clostridiales.f_Other.g_Other	0.49 ± 0.06[b]	0.07 ± 0.05[a]	0.16 ± 0.09[ab]	0.12 ± 0.10[a]	0.007
Dorea	2.92 ± 0.35[b]	0.24 ± 0.22[a]	1.29 ± 0.84[ab]	1.60 ± 1.61[ab]	0.008
Catenibacterium	0.07 ± 0.07[ab]	0.13 ± 0.14[a]	0.00 ± 0.00[b]	0.24 ± 0.29[a]	0.008
f_Lachnospiraceae.g_Other	3.11 ± 0.82[b]	0.31 ± 0.14[a]	0.92 ± 0.65[ab]	1.26 ± 1.27[ab]	0.009
Oscillospira	0.84 ± 0.29[ab]	0.65 ± 0.26[a]	2.55 ± 0.54[b]	0.89 ± 0.58[a]	0.010
Desulfovibrio	0.05 ± 0.02[b]	0.20 ± 0.23[ab]	1.31 ± 0.83[a]	0.27 ± 0.39[ab]	0.010
Ruminococcus (*f_Lachnospiraceae*)	0.27 ± 0.10[b]	0.05 ± 0.04[a]	0.28 ± 0.12[b]	0.09 ± 0.12[ab]	0.011
Roseburia	1.10 ± 0.53[b]	0.02 ± 0.02[a]	0.03 ± 0.03[a]	0.05 ± 0.05[ab]	0.012
Bacteroides	12.57 ± 6.51[a]	18.61 ± 19.55[a]	11.92 ± 3.53[a]	0.86 ± 0.83[b]	0.013
Prevotella	4.13 ± 2.69[ab]	2.07 ± 3.07[a]	2.50 ± 1.92[ab]	14.4 ± 8.29[b]	0.015
*f_Lachnospiraceae.g_*	7.97 ± 1.65[b]	4.38 ± 1.8[ab]	4.28 ± 0.63[ab]	3.03 ± 2.14[a]	0.016
*o_Bacteroidales.f_.g_*	1.38 ± 0.82[ab]	1.86 ± 2.66[a]	8.16 ± 2.85[b]	4.69 ± 4.41[ab]	0.016
Blautia	6.07 ± 1.43[b]	3.28 ± 2.44[ab]	3.02 ± 2.28[ab]	1.52 ± 1.28[a]	0.028
*f_Clostridiaceae.g_*	1.19 ± 0.28[ab]	2.10 ± 1.11[ab]	4.58 ± 2.01[a]	1.24 ± 1.32[b]	0.030
Dialister	0.74 ± 0.18[nd]	2.04 ± 1.46[nd]	2.08 ± 0.56[nd]	1.04 ± 1.51[nd]	0.032
Acidaminococcus	0.03 ± 0.03[nd]	0.03 ± 0.04[nd]	0.24 ± 0.13[nd]	0.34 ± 0.68[nd]	0.038
Collinsella	4.06 ± 0.95[ab]	15.57 ± 15.73[ab]	2.06 ± 1.12[a]	16.78 ± 10.79[b]	0.043
*f_Ruminococcaceae.g_*	19.99 ± 7.55[nd]	21.71 ± 7.62[nd]	20.86 ± 2.96[nd]	12.26 ± 2.92[nd]	0.045
Unnasigned	0.65 ± 0.18	0.82 ± 0.55	0.52 ± 0.05	0.37 ± 0.15	0.057
*f_S24.7.g_*	3.34 ± 1.69	1.12 ± 0.90	2.77 ± 2.58	5.06 ± 4.07	0.070
*f_Enterobacteriaceae.g_*	0.07 ± 0.08	0.06 ± 0.06	0.24 ± 0.17	0.21 ± 0.25	0.078
*f_Coriobacteriaceae.g_*	0.54 ± 0.52	1.48 ± 1.51	0.30 ± 0.13	0.91 ± 0.58	0.265
Ruminococcus (*f_Ruminococcaceae*)	9.28 ± 5.54	6.69 ± 7.87	6.03 ± 4.11	12.00 ± 9.46	0.270
Lactobacillus	0.07 ± 0.03	0.10 ± 0.14	0.04 ± 0.03	0.44 ± 0.89	0.745
*f_Erysipelotrichaceae.g_*	0.75 ± 0.23	1.08 ± 0.63	0.72 ± 0.24	2.63 ± 3.89	0.783

[a]Data shown correspond to basal microbiota (BMB, $t = 0$ h, $n = 5$) and substrate experiments (average of t 24, 48 and 72 h values, $n = 6$ for each substrate) ± standard deviation. Only groups with means across groups that were ≥0.1% are shown. Groups sharing an identical letter are not significantly different (Dunn's tests, $\alpha < 0.05$). The closest identified taxonomy levels are provided for those genera for which the phylogenetic information was not enough for their complete classification (denoted "g_" and/or "f_"). f_, family; g_, genus; GGMOS, galactoglucomannooligosaccharides; GGMOS_Ac, acetylated galactoglucomannooligosaccharides; nd, no differences detected by Dunn's test; o_, order; SIEM, standard ileal efflux medium.

allowed for the identification and quantification of 110 OTUs, accounting for 95.7 ± 3.5% of the total microbiota present in the samples, although for some of these genera, very low abundances were detected. Table 2 shows the relative abundances for the main 39 genera (presence across groups >0.1%). In general terms, the more abundant genera inside the lumen samples were *Collinsella*, *Bacteroides*, *Ruminococcus*, *Prevotella*, *Bifidobacterium*, *Blautia*; three unknown genera belonging to families Ruminococcaceae, Lachnospiraceae, and Clostridiaceae; one unidentified genus from Clostridiales order; and two from Bacteroidales order. A total of 33 of the 39 OTUs included showed significant differences among substrate groups (see q-values <0.05 in Table 2) based on Kruskal−Wallis test results. In addition, a linear discriminant analysis effect size (LEfSe) of samples from the fermentation experiments was carried out to find OTUs driving the main differences in bacterial profiles promoted upon the fermentation of different substrates by the elderly gut microbiota (Figure 4). Furthermore, a Random Forest analysis was applied to the genus relative abundances to find associations between the substrates used and microbiota features. The main 15 features found are summarized in Figure 5 (in all cases, the predictions were 100% correct). Additional information about these microbiota features is included in Figure S4. The most

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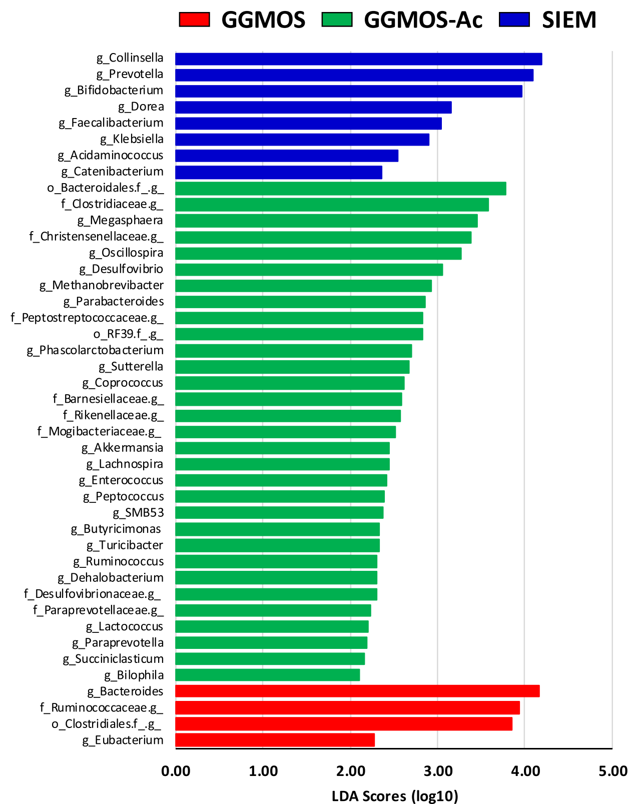

Figure 4. Linear discriminant analysis (LDA) effect size (LEfSe) score histogram (genus level) for the different *in vitro* carbohydrate interventions. For those genera for which the phylogenetic information was not sufficient to classify it to the genus level g__ or even f__, ".g__" is used, additionally indicating in which family or order respectively these OTUs fall. GGMOS, galactoglucomannooligosaccharides; GGMOS_Ac, acetylated galactoglucomannooligosaccharides; SIEM, standard ileal efflux medium; c_, class; o_, order; f_, family; g_, genus.

remarkable changes at the genus level based on genus relative abundances and/or potential importance with regard to the elderly population will be discussed in detail below.

Bacteroides Genus. Being one of the main genera, *Bacteroides* represented an average of 12.6% of the initial microbiota, a value that remained steady with both GGMOS and GGMOS_Ac (18.61 and 11.92%, respectively) and that significantly decreased in SIEM experiments to 0.86%. LEfSe analyses signaled this genus as discriminant for GGMOS fermentation and, more specifically, Random Forest identified a member of this genus (*Bacteroides ovatus*) as a feature for GGMOS_Ac fermentation (Figures 5 and S4h). The changes related to aging associated with this genus are not clear,[7] although *Bacteroides* genus members are known for being acetate producers and it was found that a part of the acetate (maybe just under specific conditions) could be converted into butyrate,[40] which has anti-inflammatory effects and which could be beneficial to counteract what is known as inflamm-aging.

Levels of *Blautia* genus were also significantly diminished with SIEM as the substrate, while no significant differences among the initial levels (6%) of this genus were observed after GGMOS or GGMOS_Ac fermentation. Particularly *Blautia coccoides* has been suggested as a target for microbiota improvement of the elderly, as it was observed to be reduced in this population and to enhance the status of gut mucosa.[4] In addition, a high presence of this genus is a strong indicator of intestinal health, and it has also been associated with a lower visceral fat area in adults (20−76 years), which might reduce the risk of metabolic-syndrome-related diseases, while lower levels have been associated with diabetes, liver cirrhosis, rectal cancer, and rheumatoid arthritis.[41]

Ruminococcus Genus. The initial abundance of this genus was 9.3%, and these levels remained practically constant (no significant differences among BMB or substrates were found). However, an unidentified genus from the Ruminococcaceae family was signaled as a discriminant for GGMOS fermentations (Figure 4). The genus *Ruminococcus* belongs

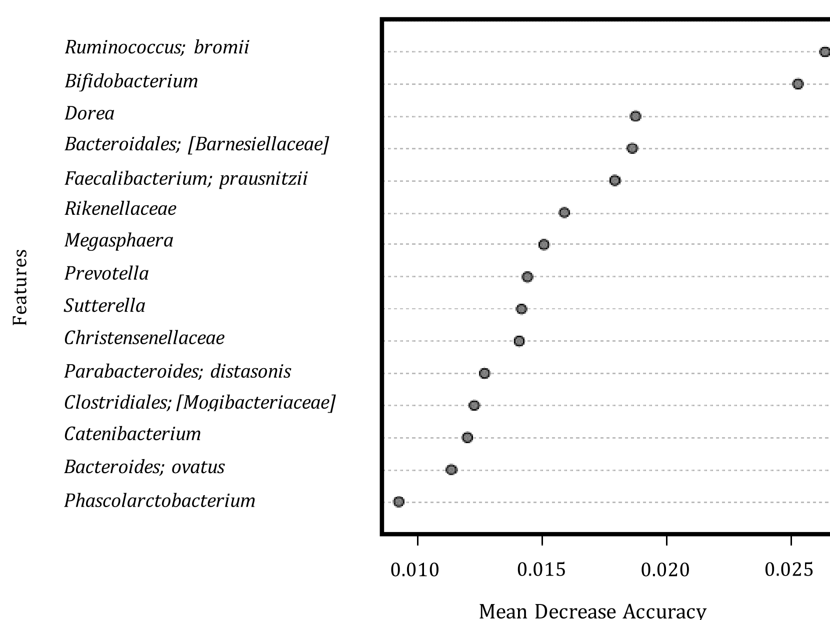

Figure 5. Mean decrease accuracy of the distinctive microbiota features (genus or, for some, even species level) revealed by Random Forest analysis for the TIM-2 *in vitro* fermentation experiments.

to the *Clostridium* XIVa cluster, which includes butyrate-producing bacteria, and several studies reported decreased levels of this cluster in the elderly.[42] Therefore, a prebiotic that can enhance its presence would also be desirable for elderly people's health improvement. A particular species, *Ruminococcus bromii*, has been indicated by Random Forest analyses as a feature for SIEM, with higher relative abundances for this substrate (Figures 5 and S4d).

Prevotella Genus. The relative abundances of *Prevotella* significantly differed between SIEM and GGMOS experiments, where relative abundances of 14.4 and 2.1% were observed, respectively, from an initial value of 4.1% in the BMB. This genus has been signaled both as a discriminant (Figure 4) and as a microbiota feature (Figures 5 and S4b) for SIEM experiments by LEfSe and Random Forest, respectively. *Prevotella* belongs to the phylum Bacteroidetes, and its abundance is characteristic of one of the three enterotypes defined in the Metahit project.[43] This genus dominates in diets rich in carbohydrate, especially fiber.[44] In this sense, SIEM is a substrate that contains diverse carbohydrates (xylan, pectin, and arabinogalactan among others), which could be related to the increase in *Prevotella* communities.

Collinsella Genus. This genus (from the phylum Actinobacteria), which has a great capacity to ferment carbohydrates and produce hydrogen and ethanol, was significantly favored in SIEM experiments (from a value of 4.1% in BMB up to a 16.8%), and the results are in accordance with those observed by Sáyago-Ayerdi et al.[18] LEfSe analyses indicated *Collinsella* as a discriminant for SIEM experiments as well (Figure 4).

Dorea Genus. GGMOS fermentation resulted in a significantly reduced presence of this genus with respect to BMB, GGMOS_Ac, or SIEM. This genus belongs to the *Clostridium* XIVa cluster, and it was signaled by LEfSe and Random Forest as discriminant and feature, respectively, for SIEM experiments (Figures 4, 5, and S4a).

Faecalibacterium Genus. Regarding this genus, the fermentation of GGMOS_Ac caused a significant reduction in its relative abundance in comparison with BMB and the other substrates and LEfSe analyses signaled it as a discriminant for SIEM experiments (Figure 4), although the particular specie *Faecalibacterium prausnitzii* was pointed out as a feature for GGMOS_Ac fermentation (lowest values, Figure S4n). This species is of great importance within the intestinal microbiota due to some interesting characteristics, such as their ability for butyrate production (and its associated positive effects earlier commented upon) and anti-inflammatory proteins, while it has also been indicated that its presence is lower in people with Crohn's disease than in healthy people.[45] It is also interesting to comment that when comparing young and centenarian microbiotas, lower Firmicutes levels (mainly the *Clostridium* XIVa cluster and *F. prausnitzii*) were observed in the latter.[36]

Bifidobacterium Genus. This genus is considered one of the most beneficial genera of the intestinal microbiota, and its presence has been observed to be reduced in elderly people[7] as well as IBS patients.[46,47] As can be observed, the basal relative abundance in the pool of elderly microbiota is found to be normal (value of 2.7%) and only SIEM substrates allowed reaching of significantly higher values (11.2%), which is in agreement with results observed previously with this substrate.[18] This is in accordance with Random Forest and LEfSe results (Figures 4, 5, and S4c), which indicate *Bifidobacterium* as a discriminant and feature for SIEM experiments.

Coprococcus spp. Its presence was significantly reduced with GGMOS and SIEM with respect to BMB, and only GGMOS_Ac maintained its presence in the lumen. This genus was observed to be depleted in depression patients,[48] and a particular specie, *Coprococcus eutactus*, is known as a butyrate producer associated with anti-inflammatory properties and the improvement of the mucosal barrier function.[49]

Oscillospira Genus. This genus was increased after GGMOS_Ac feeding, while levels were maintained with GGMOS and SIEM with respect to the BMB. This genus has been found to have a positive correlation with age in supercentenarian donors.[50]

In addition, *Methanobrevibacter* showed significantly lower values with SIEM. This genus promotes methane liberation, and, in this sense and according to the International Scientific Association for Probiotics and Prebiotics (ISAPP), prebiotics should not lead to gas distension issues after ingestion. Prebiotic fermentation must be selective and preferably not include gas formers.[12] This genus was pointed out as a discriminant for GGMOS_Ac by LEfSe (Figure 4).

Finally, *Desulfovibrio* was also included by LEfSe as a discriminant for GGMOS_Ac experiments (Figure 4), where this substrate promoted a significant rise of this genus. *Desulfovibrio* bacteria favor the liberation of H_2S, which has been reported to complicate butyrate absorption and might not be a desirable effect.[51] On the contrary, this specie has been shown to decrease *in vivo* after trans-galactooligosaccharide mixture consumption in a study performed with healthy elderly volunteers.[52]

Finally, it is necessary to state that microbiota diversity is of great importance as it has been associated with health in humans and also with a higher dietary quality.[53] Healthy elderly and elderly under medical treatment often show lower microbiota diversities.[11] In this context, α diversity was evaluated by Chao1 and Shannon indices for all of the experiments (Table 3). One-way ANOVA tests showed that significantly lower values were obtained with SIEM and GGMOS as substrates in comparison with the BMB (p <

Table 3. Bacterial Diversity at the Beginning of Fermentation and after Substrate Supplementation[b]

time (h)	substrate	Shannon	Chao1
0	BMB	6.25 ± 0.66[a*]	2160.56 ± 322.35[a*]
24	GGMOS	3.54 ± 0.57[b]	1027.17 ± 105.33[b]
	GGMOS_Ac	6.68 ± 0.25[a]	2212.44 ± 207.86[a]
	SIEM	4.43 ± 0.45[b]	1188.81 ± 9.69[b]
48	GGMOS	4.39 ± 0.21[b]	1202.9 ± 175.51[b]
	GGMOS_Ac	6.66 ± 0.09[a]	2030.5 ± 83.36[a]
	SIEM	4.44 ± 0.47[b]	959.37 ± 173.42[b]
72	GGMOS	5.08 ± 0.76[a]	1297.12 ± 365.49[b]
	GGMOS_Ac	6.34 ± 0.04[a]	1740.54 ± 41.66[ab]
	SIEM	5.68 ± 0.17[a]	1556.64 ± 84.05[ab]

[a]To avoid repetition, [a]* indicates the letter code obtained in all multiple comparisons. [b]Data presented corresponds to average ± standard error; rarefied to 16 500 sequences per sample. Different superscripts show significant differences among BMB (t = 0 h) and substrates at each fermentation time (24, 48, and 72 h) according to Duncan's *post hoc* tests ($p < 0.05$). BMB, basal microbiota; GGMOS, galactoglucomannooligosaccharides; GGMOS_Ac, acetylated galactoglucomannooligosaccharides; SIEM, standard ileal efflux medium.

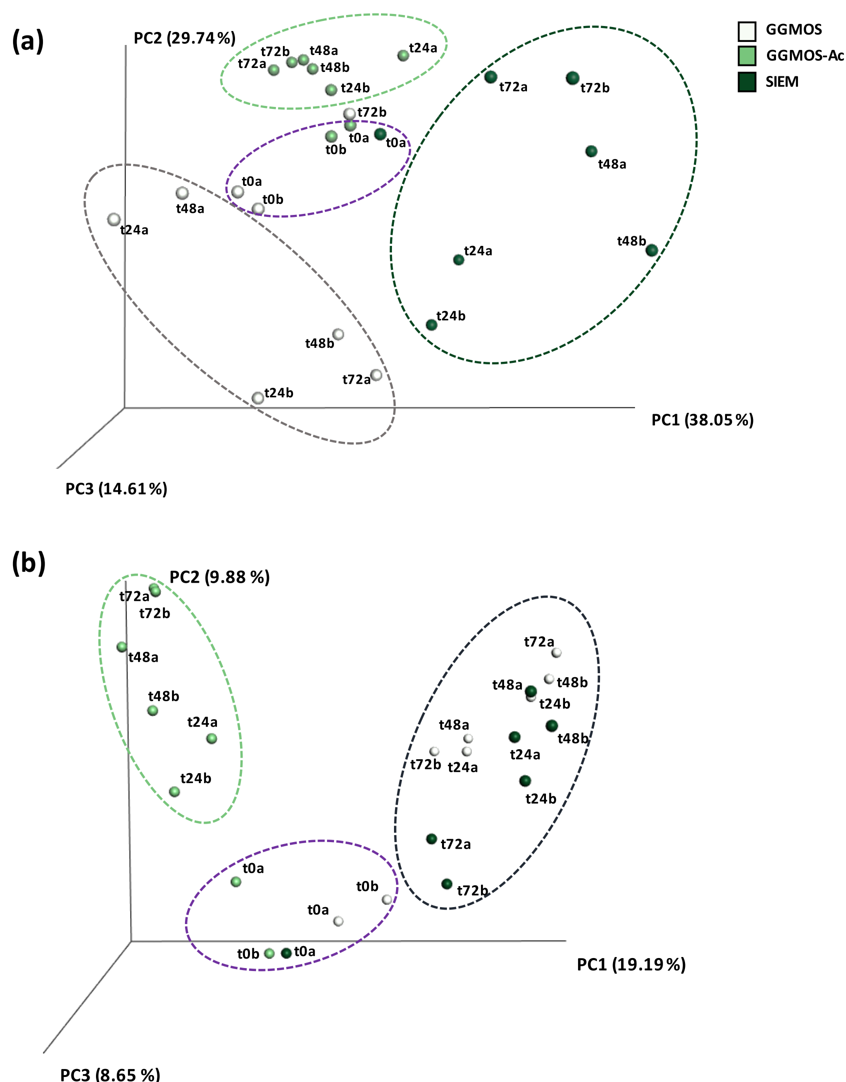

Figure 6. Principal coordinate analysis (PCoA) plot of microbiota composition measured throughout the experiments performed in the TIM-2 (n = 2 for each test compound). (a) Weighted PCoA and (b) unweighted PCoA. Colors encode the corresponding test substrate (see the figure legend). Coding numbers next to the spheres describe the sampling time points (24, 48, or 72 h), and the letters "a" or "b" indicate the corresponding duplicate experiment. The dotted circles delineate the different substrates, as well as the clustering of the t = 0 h samples (purple). GGMOS, galactoglucomannooligosaccharides; GGMOS_Ac, acetylated galactoglucomannooligosaccharides; SIEM, standard ileal efflux medium.

0.05), while GGMOS_Ac allowed maintaining comparable values to those obtained at t = 0 h.

Principal Coordinate Analysis (PCoA). To assess the effects of the different *in vitro* fermentation experiments on the gut microbiota from elderly donors, a PCoA with respect to the weighted and unweighted UniFrac distances was also carried out with the OTU output from QIIME analysis, and the plots obtained are shown in Figure 6. The weighted PCoA (Figure 6a) shows that PC1 explained 38.05% of the total microbiota composition variability found throughout the experiments, whereas PC2 and PC3 explained 29.74 and 14.61%, respectively. Samples clustered separately by substrate fermented, and practically all of the duplicate experiments for each substrate were clearly located in their cluster, demonstrating that experiments performed in this artificial colon are very reproducible even though multiple variables were involved in the functioning of the continuous *in vitro* fermentation (Figure 1). It was also confirmed that the samples corresponding to the initial microbiota pool (t = 0 h samples) clustered closely before being modified by the different carbohydrates (purple dotted circle). The unweighted PCoA (Figure 6b) shows that samples from experiments with SIEM and GGMOS shared the same cluster and were clearly separated from GGMOS_Ac samples, suggesting that SIEM and GGMOS promoted similar effects in the gut microbial structure of the elderly donors, while the GGMOS_Ac substrate promoted distinct effects, as discussed above.

Effects on Microbiota Metabolism: Organic Acids Production. The colonic fermentation of nondigestible carbohydrates results in the production of a mixture of organic acids, with the SCFA (acetic, propionic, and butyric acid) being the most abundant. The cumulative production (mmol) of the main SCFA along the test period (from 0 to 72 h) is shown in Figure 7a−c. Notable differences were found in the evolution of the SCFA production during experiments with the different substrates. A lag period of 24 h was observed in experiments carried out with GGMOS, followed by a steady stage (constant slope in acid production) where the production of acetate, propionate, and butyrate acids reached values of up to 64.4 ± 3.6, 28.9 ± 2.1, and 31.2 ± 2.7 mmol at

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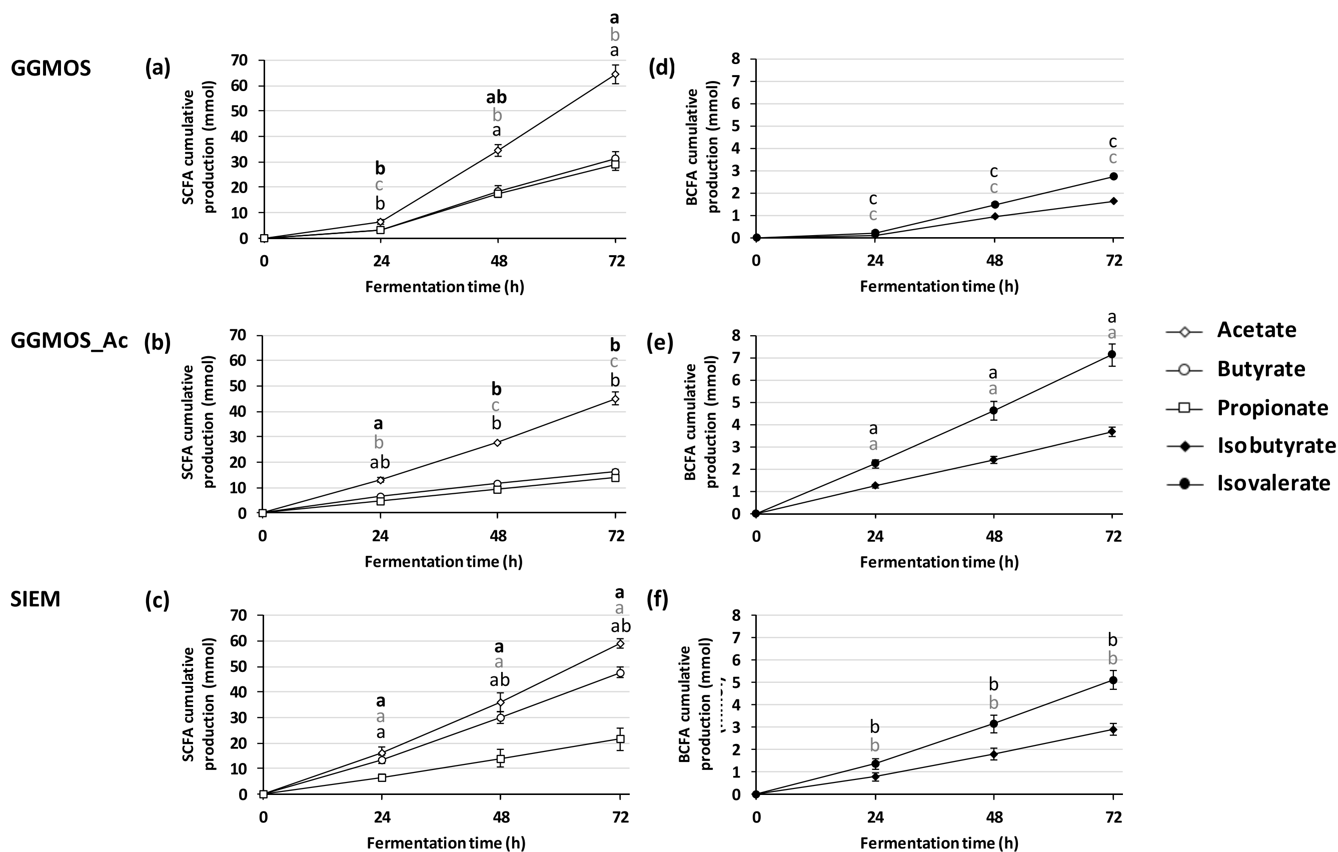

Figure 7. Average of the cumulative production (expressed in mmol) of the main SCFA (acetate, propionate, and butyrate) and BCFA (isobutyrate and isovalerate) during TIM-2 fermentation experiments using GGMOS, GGMOS_Ac, or SIEM as carbohydrate sources ($n = 2$). The amount of SCFA and BCFA was artificially set to zero at $t = 0$, and bars indicate standard deviations for duplicate assays. Superscripts (a, b, or c) denote significant differences among substrates in SCFA or BCFA at each fermentation time (24, 48, or 72 h) assessed by Duncan's *post hoc* tests ($p < 0.05$); bold, gray, and black letters in figures (a), (b), and (c) correspond to acetate, butyrate, and propionate, respectively, while black and gray letters correspond to isobutyrate and isovalerate in graphs (d), (e), and (f). BCFAs, branched-chain fatty acids; GGMOS, galactoglucomannooligosaccharides; GGMOS_Ac, acetylated galactoglucomannooligosaccharides; SCFAs, short-chain fatty acids; SIEM, standard ileal efflux medium.

72 h, respectively. On the contrary, the SCFA production throughout *in vitro* fermentation with GGMOS_Ac and SIEM showed a linear increment during the whole test period. When comparing the total production of the aforementioned SCFA, GGMOS_Ac showed the lowest values (75.3 ± 3.2 mmol), while significantly higher productions (Duncan's test $p < 0.05$) were reached with GGMOS and SIEM as carbohydrate sources (124.4 ± 8.4 and 128.4 ± 8.2 mmol, respectively). These results are in agreement with those obtained by Sáyago-Ayerdi et al.,[18] where 128.8 mmol of these major SCFAs was produced after SIEM intervention. In addition, when only taking into account the fermentation period between 24 and 72 h (where a steady SCFA production was observed for all of the substrates), higher average productions of acetate, propionate, and total SCFA were obtained with GGMOS in comparison with SIEM (respectively, 1.21 ± 0.05, 0.53 ± 0.08 mmol/h, and 2.33 ± 0.11 with GGMOS *vs* 0.89 ± 0.10, 0.31 ± 0.00, and 1.92 mmol/h with SIEM), which may be due to the availability of unconverted substrate in the GGMOS experiment. This suggests that, even though the microbiota required more time to utilize GGMOS as a carbon source, the total cumulative production of these major SCFAs could reach similar values at $t = 72$ h to those obtained with SIEM and significantly higher than the ones achieved with GGMOS_Ac. The lower SCFA production achieved in experiments with GGMOS_Ac could be related to the lower content of hexose in this substrate, in comparison with GGMOS or SIEM, as just 60.3% w/w of the substrate corresponds to hexoses, with almost the rest being acetyl groups (Table 1). Ouyang and co-authors[54] also observed differences in SCFA production depending on the deacetylation degree of konjac glucomannan polysaccharides, achieving less SCFA concentrations with increased deacetylation degrees. Moreover, in this work, GGMOS_Ac appear to be metabolized by different bacterial groups, which might have promoted the production of different metabolites as previously discussed.

On the other hand, the average molar distribution of acetate/propionate/butyrate was 52:23:25 for GGMOS, 60:18:22 for GGMOS_Ac, and 46:17:37 with SIEM; therefore, GGMOS fermentation gave as a result SCFA mixtures richer in propionate, GGMOS_Ac in acetate, and SIEM in butyrate. The SCFA ratios found for GGMOS_Ac were very similar to those achieved after an intervention adding 7.5 g/day of mango peel and SIEM in the study performed by Sáyago-Ayerdi et al.[18] mentioned above. The higher acetate ratios observed upon GGMOS_Ac fermentation might be due to the greater content in acetyl groups of this carbohydrate source, which would support the hypothesis of esterification as a suitable mechanism to increase acetic acid generation without a rise in the dose of fermentable carbohydrates (that also could

increase the gas generation). Actually, the production of acetate from GGMOS_Ac constituted around 70% of that achieved with GGMOS, whereas the propionic and butyric acids corresponded to values close to 50% of those of GGMOS, which is in accordance with the lower hexose availability for the gut microbiota in GGMOS_Ac compared to GGMOS fermentations (11.1 *vs* 18.4 g). In addition to the main SCFA, other organic acids were produced in minor amounts: branched-chain fatty acids (BCFAs; isobutyrate and isovalerate) from protein fermentation and other acids considered as intermediate products (such as succinate, lactate, or formate). Figure 7d−f shows the BCFA production in the TIM-2 experiments (average ± SD), where a lag phase is again observed in experiments with GGMOS, which led to approximately half of the BCFA production than that observed with SIEM, which in both cases are significantly lower than those obtained with GGMOS_Ac. BCFAs have been identified as a marker of protein fermentation and pointed out as an indicator of carbohydrate availability for the microbiota in the TIM-2.[55] This result suggests that due to the lower carbohydrate/protein ratio in the GGMOS_Ac fermentations (43% w/w of the substrate corresponded to acetyl groups and not to sugars, and just the protein content in the SIEM without the carbohydrate medium needs to be taken into account as the protein content GGMOS-Ac is lower than 0.01% w/w), there was an early switch from carbohydrate to protein fermentation, a hypothesis supported by the higher lumen pH values and also by the absence of NaOH consumption, which is normally used to keep the pH at 5.8 (data not shown). Regarding the cumulative production of other organic acids, as can be seen in Table 4, lower productions were, in general, observed. After 72 h of fermentation, significantly higher productions of valerate were found with GGMOS_Ac and, in contrast, GGMOS led to the lowest production, while no significant differences across groups were found for succinate, lactate, formate, or caproate. In addition, it was observed that the ratio of SCFA/total organic acid was almost constant throughout the fermentation time, varying in the range 0.9−0.93 for GGMOS, 0.83−0.85 for SIEM, and 0.74−0.75 with GGMOS_Ac. Therefore, it can be concluded that GGMOS were the most selective substrates for increasing the SCFA production in elderly microbiota, which would have a great interest given that the capacities for the production of these acids decline with age.[56] The major SCFAs did not show significant correlations with specific OTUs, although some correlations were observed between some of the minor metabolites and some OTUs (Table S2). The main positive correlations involving different OTUs and the intermediate acids formate and valerate (in some cases both) were found, although, particularly, the genus *Acidaminococcus* and an unknown genus from the Enterobacteriaceae family also showed a significant correlation with the BCFA (isovalerate and isobutyrate) cumulative production.

Table 4. Cumulative Production of Other Organic Acids (Expressed as mmol ± SD) after 72 h of *In Vitro* Fermentation[a]

product	GGMOS	GGMOS-Ac	SIEM
succinate	2.79 ± 0.15	0.36 ± 0.30	4.23 ± 2.63
lactate	0.09 ± 0.02	0.24 ± 0.05	0.23 ± 0.04
formate	0.88 ± 0.13	2.19 ± 0.70	1.17 ± 0.74
valerate	3.75 ± 0.36[c]	10.73 ± 0.09[a]	6.33 ± 0.28[b]
caproate	2.08 ± 0.11	3.04 ± 0.41	5.73 ± 2.58

[a]Superscripts indicate the existence of significant differences among substrates at sampling time points 24, 48, or 72 h (one-way ANOVA followed by Duncan's *post hoc* tests, $p < 0.05$). Groups sharing the same letter are not significantly different. GGMOS, galactoglucomannooligosaccharides; GGMOS-Ac, acetylated galactoglucomannooligosaccharides; SIEM: standard ileal efflux medium.

Taking into account the effects on the gut microbiota composition (mainly the lower Proteobacteria percentages and the increase in the F/B ratio) and the profile and production of metabolites (higher SCFA production and the lowest BCFA cumulative productions), we conclude that GGMOS would have a greater potential to counteract the undesirable alterations normally found in elderly microbiota than GGMOS-Ac. However, the acetylation of these substrates (GGMOS_Ac) promoted positive outcomes such as higher microbial diversities after fermentation, suggesting further studies are needed to evaluate additional parameters such as optimal acetylation degree, gas production, and consumption rate. This study supports the interest of new research in the prebiotic field where compounds with a prebiotic potential could be employed also as "carriers" of chemical substituents with interesting properties (such as acetyl groups) to enhance their native prebiotic attributes, but further research will contribute to a deeper insight into the effects of mannan-derived oligosaccharides and their degree of acetylation in the health of the elderly.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c02225.

Additional tables and figures showing HPSEC elution profiles of purified GGMOS (Figure S1), FTIR partial spectra of purified GGMOS and GGMOS_Ac (Figure S2), MALDI-TOF MS spectra of purified GGMOS (Figure S3), Random Forest normalized abundances of the top genera that differentiate between the media used (Figure S4), MALDI-TOF MS GGMOS oligomer profile (Table S1), correlations between OTUs and organic acids (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

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