Effect of beta-glucan supplementation on cystic fibrosis colonic microbiota: an *in vitro* study

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Impact:

- Current evidence supports assessing the effect of prebiotics on modifying cystic fibrosis microbiota.

- The effect of beta-glucan supplementation was evaluated in a controlled dynamic *in vitro* colonic ecosystem.
- Beta-glucan supplement improved diversity in cystic fibrosis colonic microbiota.
- The treatment showed increased abundance of *Faecalibacterium* and *Akkermansia* in cystic fibrosis.
- New evidence supports the mechanism of a prebiotic for future clinical studies.

Abstract

Background: Children with cystic fibrosis (CF) present with dysbiosis in the gut, and current evidence impedes robust recommendations on the use of prebiotics. This study aimed at establishing the prebiotic potential of a commercial beta-glucan on the *in vitro* colonic microbiota of a child with CF compared to a healthy counterpart (H).

Methods: A dynamic simulator of colonic fermentation (twin-SHIME® model) was set up including the simulation of the proximal (PC) and distal colon (DC) of the CF and the H subjects by colonizing the bioreactors with faecal microbiota. During two weeks the system was supplied with the study beta-glucan. At baseline, during treatment and post-treatment, microbiota composition was profiled by 16S rRNA and short-chain fatty acids (SCFA) production was determined by GS-MS.

Results: at baseline, *Faecalibacterium*, was higher in CF' DC than in the H, along higher *Acidaminococcus* and less *Megasphaera* and *Sutterella*. Beta-glucan supplementation induced increased microbiota richness and diversity in both subjects during the treatment. At genus level, *Pseudomonas* and *Veillonella* decreased, while *Akkermansia* and *Faecalibacterium* increased significantly in CF.

Conclusion: the supplementation with beta-glucan suggests positive results on CF colonic microbiota in the *in vitro* context, encouraging further research in the *in vivo* setting.

1. INTRODUCTION

The intestinal disease in Cystic Fibrosis (CF) and pancreatic insufficiency entails a wide spectrum of alterations that lead to maldigestion and malabsorption of nutrients,

which are related to nutritional status, quality of life and disease prognosis and survival[1]. Among them, the obstruction of the pancreatic duct is responsible for the decreased secretion of digestive enzymes and low pH, and hepatic alterations account for reduced bile salts in the lumen [2]. While the mechanisms are still not well defined, the altered conditions in the small intestine, along with the frequent use of antibiotics for the lung disease, seem to be responsible of dysbiosis in the colonic microbiota of children with CF [3]. The altered microbiota in this context refers to decreased microbial diversity [4], unbalanced beneficial/pathogenic bacteria[5] functional alterations in fatty acids metabolism [6].

In recent years, targeted dietary interventional approaches with -biotics family (probiotics, prebiotics and synbiotics) to modulate dysbiosis in CF have been described[7]. Despite current knowledge allows for affirming that probiotic supplements improve pulmonary exacerbations, there are scarce reports on its impact on colonic microbiota [4]. Variable responses to probiotics found in clinical trials has led to the conclusion that personalized approaches that consider the microbiota characteristics of the individuals should be targeted to define supplementation with prebiotics, especially in the multi-factorial context of CF [7]. Regarding the use of prebiotics, the lack of studies on their potential to improve gut microbiota is limiting the degree of evidence for recommending their use. On the other hand, galacto-oligosacchararides and fructo-oligosaccharides, which are some of the most common prebiotics used in the infant population via infant formulas, have not confirmed an apparent beneficial role [8]. However, less is known about other types of compounds with prebiotic potential that have been studied in adults with CF [9], or others, such as beta-glucans, that have not been assessed yet.

In this sense, the use of *in vitro* models to simulate digestion may be a helpful approach to identify the impact of a prebiotic compound on modifying the colonic

microbiota of a subject. Despite the limitations of a non-biologic system in the controlled context of this type of experiment, confounding factors such as diet, use of antibiotics or any other variable can be omitted, making *in vitro* models a suitable approach as screening tools.

Therefore, the aim of the present study was to assess the potential of supplementation with beta-glucan as a prebiotic on modifying the colonic microbiota of a child with CF as compared to healthy control, using for the first time in this context, a dynamic *in vitro* digestion and colonic fermentation model.

2. METHODS

2.1.Study design

The dynamic Simulator of Human Intestinal Microbial Ecosystem (SHIME®) (Prodigest®, Gent, Belgium) was used to simulate colonic fermentation of two children (one with CF and one healthy control) using their individual faecal inoculums to colonise the respective bioreactors within the equipment. As substrate, a nutritional medium with beta-glucan was supplemented to the equipment daily for 2 weeks. The beta-glucan (commercial name: BetaGlucan Complex) from fungal origin was supplied by Lamberts laboratories (Madrid, Spain). The supplement was composed by 1-3/1-6 beta-glucan, yeast (*Saccharomyces cerevisiae*), selenium, vitamin D3 and vitamin C. At different time points aliquots were taken to analyse subject-specific changes in colonic microbial populations, production of short chain fatty acids (SCFA) and other metabolism indicators such as lactate and ammonia.

The volunteer with CF was an 8-year-old child, with confirmed diagnosis of CF by a sweat chloride ≥ 60 mEq/L and the presence of 2 disease causing mutations in the CFTR gene, confirmed diagnosis of exocrine pancreatic insufficiency by faecal elastase values

(FE1) < 200 mcg/g of faeces and treatment with pancreatic enzyme replacement therapy (PERT). The child had had no acute infections or acute abdominal pain, was not on a CFTR modulation therapy or had used antibiotics in the last 2 weeks before the experiment. The healthy subject was an 8-year-old child with no pathologies or conditions. Faecal samples were collected with the consent of the legal guardians of the volunteers.

Protocols and the study were approved by the ethical committees of Universitat Politècnica de València (Valencia, Spain) (Ref. P09_24_11_2021) and Hospital La Fe (Valencia, Spain) (Ref. 2021-111-1).

2.2.Faecal collection and inoculum preparation

Faecal samples (40 grams) from the volunteer children were fresh collected and kept in refrigeration at 8 ºC and anaerobiosis conditions with the use of anaerobiosis bags (Thermo Scientific™ Oxoid AnaeroGen). Samples were processed in a period of less than 24 hours before the experiment. The faecal inoculums were obtained from each sample the day after the collection. First, the faecal material was mixed in anaerobic phosphate buffer in 20% (w/v) proportion and homogenized for 10 minutes in a Stomacher. The resulting mixture was poured into a 50 mL falcon tubs and centrifuged during 2 minutes at 500 g-force. Then, the supernatant was collected and considered as the faecal inoculum, from which 5 mL per 100 mL of the volume of nutritional medium were inoculated in the corresponding bioreactors of the equipment.

2.3.*In vitro* colonic fermentation using the SHIME®

A twin-SHIME® configuration was set to carry out the experiment, which allows for reproducing colonic fermentation of two subjects simultaneously.

The twin-SHIME setting consisted of six double-jacketed vessels or bioreactors (three per subject) simulating: (1) the stomach-duodenum, (2) the proximal colon and (3) the distal colon. The vessels were continually agitated with a magnetic stirrer and warmed to 37 ºC. Nitrogen flow maintained the anaerobic environment in the reactors and the pH was monitored using a pH-meter and automatically adjusted with NaCl (0.5 N) and HCl (0.5 N)[10]. Each compartment of the colon (proximal and distal) was filled with a specific volume of nutritional medium. The stomach and intestinal conditions as well as the pancreatic juice (6 g/L Oxgall, 12.5 g/L $NaHCO_3$ and 0.9 g/L of pancreatin) were simulated as previously described[11]. Prior the experiment, 25 and 40 mL of two faecal inoculums were inoculated to the proximal and distal colon, respectively.

The experimental protocol included two weeks for the stabilisation of the microbiota from the faecal inoculums in the bioreactors, followed by other two control weeks, considered as the baseline microbiota. After this period, the beta-glucan supplement (250 mg per day) was incorporated to the system along with the nutritional medium that fed the system daily during two weeks, considered as the treatment period. This period was followed by two more weeks with the same conditions as in the control period, i.e., supplying to the system nutritional medium, but with no beta glucan (post-treatment). The nutritional medium PDNM002B was acquired from Prodigest® (Gent, Belgium).

Two aliquots from the proximal (PC) and distal colon (DC) bioreactors were taken after seven and fourteen days of each period (baseline, treatment and post-treatment), and samples were stored at -80℃ until analysis.

2.4.Microbiota composition by 16S rRNA amplicon gene sequencing

Microbiota composition was analysed once a week in all the study periods (baseline, treatment and post-treatment) from the PC and DC (i.e., two aliquots per period and per colonic region) by 16S rRNA amplicon gene sequencing. Total DNA was extracted from

the SHIME samples using the Stool DNA Isolation Kit from Norgen Biotek Corp® (Ontario, Canada), following the manufacturer protocol and recommendations. Final yield of the extracted DNA was determined by fluorometry (Qubit fluorometer, Invitrogen Co., Carlsbad, CA). The microbiological analysis was performed by amplification with specific primers of the V3-V4 regions of the 16S rRNA using Illumina. Amplicons were checked with a Bioanalyzer DNA 1000 chip and libraries were sequenced using a 2 × 300 bp paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). The sequences obtained by sequencing on the Illumina MiSeq platform (2x300bp) were filtered for subsequent analysis. The fastq files were filtered on the basis of quality (removal of low quality nucleotides at the 3' end, and remove windows 5 nt with a low average quality) and length (removal of sequences with less than 100 pb) with with prinseq[12], and the paired-end reads with a minimum overlap of 30 bp were joined using Fastq-join. The sequences were also trimmed of primers and distal bases and singletons were removed with USEARCH v11. ASVs mapping to the human genome (GRCh38) using the Burrow– Wheeler Aligner in Deconseq v0.4.3 were filtered out. Reads were denoised into zero-radius operational taxonomic units (ZOTUs) and chimeras were filtered with UNOISE3. Taxonomic assignment of ZOTUs was performed in QIIME 2 v2018.2 using the QIIME 2 feature classifier plugin and the Ribosomal database project (RDP). Finally, Decontam R package was used to compare prevalence of ASVs in real samples and negative controls, identifying contaminant ASVs that were removed from downstream analyses. Microbiota richness and diversity were estimated through the calculation of Chao1 and Shannon indices for each rarefied sample using the phyloseq R package.

2.5. Metabolic activity: Short-chain fatty acids (SCFAs), ammonia and lactate

SCFAs were analysed from aliquots taken at the same time points as for microbiota analyses, by chromatography (GC-MS) according to the protocol adopted by Zheng et al., (2013)[13]. Analytical calibration lines were prepared for the quantification of the volatile acids of interest: acetic acid (AA), propanoic acid (PA), butyric acid (BA), valeric acid (VA), isovaleric acid (IVA) and isobutyric acid (IBA) ranging from 0 to 25 mM. The samples (200 µL) were mixed with 800 µL of the internal standard extraction solution (5 µmol 3-methylvaleric acid) and vortexed. Then, 1 mL of diethyl ether was incorporated along with a spoon tip of Na_2SO_4 to remove any traces of water in the extract before injection, allowing for 10 min of reaction, and then centrifuged 2000 g-force for 5 min. The supernatant was injected in the equipment Agilent GC7890B-5977B GC-MS with a multipurpose sampler (Gerstel MPS) (IATA, Valencia, Spain) with an Agilent DB-FATWAX column (30 m x 0.25 mm x 0.25 µm) operated in split mode (20:1). The oven temperature program was 100 °C for 3 min, ramped to 100 °C at a rate of 5 °C /min, to 150 °C for 1 min to 200 °C at a rate of 20 °C/ min, and finally held at 200 °C for 5 min. Helium was used as carrier gas at a flow rate of 1 mL/min with an inlet temperature of 250 °C, the injection volume was 2 µL. Results were expressed in millimolar concentration (mM).

The concentration of ammonia was quantified using the kit Ammonia of R-Biopharm (Darmstadt, Germany) and by a spectrophotometer (UV/vis, Beckman Coulter), following the instructions and recommendations of the manufacturer. Results were expressed in millimolar concentration (mM). The concentration of lactate was quantified using the Lactate Assay kit of Sigma Aldrich® (Missouri, EE. UU) and a spectrophotometer Victor 3 Multilabel Counter (model 1420- 051, Perkin Elmer, Cleveland, Ohio) following the instructions of the manufacturer. Results were expressed in micromolar concentration (µM).

2.6.Statistical analysis

Data on relative abundance at phylum and genera levels, Shannon index and CHAO1, lactate and ammonia and SCFA, were summarised as mean and standard deviation or median and first and third quartiles for descriptive analysis. For all the results, two duplicates were obtained. The Graphpad Prism (v 8.0) was used for inference analysis. Two-way ANOVA analyses were applied to explore differences between study periods (baseline, treatment and post-treatment), subjects (CF and H) and colon sites (proximal colon and distal colon) in terms of relative abundance of phylum and genera (data were log-transformed), richness and diversity, and metabolite production. Finally, correlations between bacteria populations at genus level and metabolite production (SCFA and lactate and ammonia) were analysed as a soleXY matrix, regardless the subject, colon site or study period. Statistically significant differences were considered when p-values < 0.05.

3. RESULTS

3.1.Effect of prebiotic administration on colonic microbiota populations

Significant differences between CF and H microbial scenarios were detected at the three stages of the study. In baseline, the only difference was higher Actinobacteriota in CF than in H, in the PC ($p = 0.0451$), but the treatment period with the beta-glucan supplement induced more changes at this taxonomic level. Both in treatment and post-treatment the differences were found in the DC: Verrucomicrobiota became higher in CF ($p<0.001$), and Actinobacteriota was maintained higher, while Desulfobacteriota increased in H ($p<0.001$) (**Figure 1**). Because of the supplementation, other within-subject changes occurred, such as the increase of Proteobacteriota in the PC of both subjects after the treatment. Also, in the DC of the CF subject, Actinobacteriota ($p=0.0268$) and Desulfobacteriota ($p<0.0001$) decreased during treatment, while

Verrucomicrobiota increased ($p<0.0001$). In contrast, in the DC of the H subject, a decrease in Verrucomicrobiota was detected during treatment with the beta-glucan. Regarding the different regions of the colon, specific patterns were found between PC and DC in the CF and the H subject. In both subjects Desulfobacteriota ($p<0.0001$) and Verrucomicrobiota ($p<0.0001$) were found to be higher in the DC than in the PC, in all the stages. In addition, in both subjects, Proteobacteriota was significantly lower in the DC ($p<0.0024$) during treatment and post-treatment ($p<0.0054$).

Focusing on genus level (**Figure 2**), more specific patterns of microbiota composition were obtained, with detected differences between subjects, colon sites and treatment stages. Results showed that overall *Bacteroides* and *Lachnoclostridium* were the most abundant. In the DC of CF most of the changes related to the effect of the beta-glucan occurred. Concretely, *Veillonella* and *Pseudomonas* decreased ($p<0.0001$) from baseline to post-treatment, while *Akkermansia* ($p<0.0001$) and *Faecalibacterium* increased($p<0.0001$). On the other hand, a significant increase of *Akkermansia* members in the CF' DC after the treatment ($p<0.0001$) was documented. In addition, in the H subject, *Akkermansia* decreased during the treatment, but it seemed to increase again once the supplementation of beta-glucan ceased. When focusing on the PC of the CF subject, *Acidaminococcus*, and *Pseudomonas* decreased ($p<0.0001$). In the H subject, the changes in the PC were related to decreased *Bacteroides* in the post-treatment ($p=0.0015$) and *Lachnoclostridium* in the treatment ($p=0.0169$); and in the DC, *Faecalibacterium* decreased in the post-treatment ($p<0.0001$).

The specific differences related to subject type were higher *Acidaminococcus* ($p<0.0001$) and lower *Alistipes* ($p<0.0001$), *Megasphaera* and *Sutterella* in CF than in H, both in PC and DC, at the three stages of the study. In addition, during post-treatment, *Faecalibacterium* was higher in the PC of the CF subject, while *Veillonella* was lower

($p<0.0001$); and in the DC, during treatment and post-treatment, *Veillonella* was higher in H than in CF, and *Akkermansia* was higher in CF. *Bifidobacterium*, was significantly lower in CF than in H ($p<0.0001$), in the DC, during post-treatment.

3.2.Effect of prebiotic administration on richness and diversity

The reported changes at phylum and genus level can be contrasted against the analysis of richness (Chao1) and diversity (Shannon Index) in **Table 1**. As observed, in all stages of the study (baseline, treatment and post-treatment) and both in PC and DC, the CF subject had significantly lower values ($p<0.0001$). As observed in the table, both diversity and richness were higher in the DC than in the PC of both subjects and at all stages of the study, except in the case of the treatment stage for the CF subject, when both colon sites obtained equivalent values of the Shannon index. The significantly higher richness in the DC of the CF subject during treatment and post-treatment could be related to the fact that "others" increased at genus level. This increasing richness was also observed in the H subject, but was not found to be significantly different.

3.3. Impact of prebiotic administration on metabolic activity

The metabolic activity of the microbiota was monitored along the study in terms of lactate and ammonia and short chain fatty acids (SCFA). Our results indicate no significant changes along the treatment periods (**Table 2**). However, increased ammonia in the DC compared to the PC, both in the CF and the H subject were observed. Focusing on lactate, high levels, especially during the treatment period of CF and H subject in the PC, were obtained. The supplementation with beta-glucan led to an increase of this metabolite in the PC during the treatment period, in both subjects. In addition, lactate was

found to be higher in the H than in the subject with CF, in the treatment and post-treatment periods.

On the other hand, the total production of SCFA ranged between 70 and 100 mM (**Figure 3**). The majoritarian was acetic acid (AA), followed by butyric (BA) and propionic (PA). Valeric acid (VA) was only detected in the DC of the H subject at all the stages of the simulation, and also in the PC of this same subject only during treatment. Isovaleric acid (IVA) was present in smaller amount in all the cases, while caprionic (CA) and isobutyric (IBA) were in the smallest proportions. No differences were found between subjects, except for total SCFA in the DC during the treatment period, when the values were significantly higher in the H subject. In addition, we found that total SCFA production was significantly higher in the DC than in the PC at all of the stages of the study.

3.4. Correlation between gut microbiota and metabolic activity

The correlation analysis between SCFA and other metabolites with specific bacterial genera revealed several positive or negative associations (**Figure 4**). As for the statistically significant correlations, *Bacteroides, Veillonella, Akkermansia, Alistipes, Sutterella* and *Faecalibacterium* showed positive correlations at least for one metabolite, being valeric acid (VA) common in all of them, and *Alistipes* showing the highest r value ($r=0.789$; $p<0.0001$). *Bacteroides* had the most significant and positive correlations with all the SCFA except for butyric acid (BA) and isobutyric acid (IBA). The highest correlation with production of BA was found for *Faecalibacterium* ($r=0.601$; $p<0.0019$). In contrast, *Lachnoclostridium* (e.g., with VA $r=-0.706$; $p=0.0001$), *Acidaminococcus* (e.g., with VA $r=-0.605$; $p=0.0017$) and *Enterococcus* (e.g., with propionic acid (PA) $r=-0.530$; $p=0.0077$) were correlated with decreased production of metabolites.

Pseudomonas also showed decreasing tendencies, but with no statistical significance apart than that obtained for ammonia.

Acidaminoccus, despite was found to led to significantly lower production of a wide range of metabolites, showed a positive (though not significant) correlation with the production of acetic acid (AA). *Veillonella* was positively correlated with the production of lactate (r=0.673; p=0.0008). *Faecalibacterium* showed the highest correlation with BA and it was correlated with other metabolites such as VA (r=0.491; p=0.0149), caproic acid (CA) (r=0.431; p=0.0357), lactate (r=0.495; p=0.0311) and ammonia (r=0.626; p=0.0018), being along with *Bacteroides*, the genus showing the highest number of positive correlations. Regarding *Akkermansia*, a significant and positive correlation with the production of PA was found (r=0.499; p=0.0193).

4. DISCUSSION

This study allowed for establishing differences in colonic microbiota between a child with CF and a healthy control in the environment of a dynamic *in vitro* simulator of colonic fermentation SHIME®, when exposed to a supplement based on beta-glucan during two weeks. Changes in colonic microbiota composition and metabolism were detected in terms of treatment period and colonic regions.

Some comparisons in microbiota phylum with previous studies using the SHIME can be made. For instance, Proteobacteria showed to be lower in the DC than in the PC in both subjects, which is in accordance with Huang et al. (2023) who used the SHIME in the same PC-DC configuration to study the effect of fibers[14]. In contrast, this result is opposed to the findings by Van den Abbeel et al. (2010)[15]. However, similarities with this study are observed, including increased Verrucomicrobiota in the distal part of the colon, along with decreased Actinobacteriota and Bacteroidota showing no differences. A

consideration when comparing both studies is that Van den Abeel et al. applied a triple SHIME® configuration, meaning that three specific colon sites were simulated.

At genus level, the supplementation induced most of the changes in the DC of the CF subject. The reduction of both *Veillonella* and *Pseudomonas* can be considered as positive outcomes derived from the use of the beta-glucan. *Veillonella* is known to be increased in the gut of CF patients, especially in the youngest [4,16,17], as shown in this study. The origin of *Veillonella* in the CF gut might be related to its presence in the lungs, where its abundance can reach up to 50% in children with CF [18]. Some hypotheses point to the swallowing of the mucus produced by the lungs as a mechanism of the increased presence of pathogenic bacteria in the CF gut [19]. Some species belonging to the *Veillonella* genus, such as *Veillonella spp*., are associated with gastrointestinal symptoms in patients with inflammatory bowel disease [20], but up to now, no evidence exists for CF. Related to *Pseudomonas*, its presence in the lungs is also majoritarian [18], and the increased presence in the colon could be also related to the lung-gut pathway via swallowing of the mucus. A study in mice suggested that the presence of *P. aeruginosa* in the gut, which is the most common specie among patients with CF [21], affects taxonomic microbiota composition and diversity.

On the other hand, the increase of *Akkermansia* members in the CF' DC after the treatment is also considered a positive finding, which was also detected at phylum level (Verrucomicrobiota). The relative abundance was found to increase >5% during the treatment period, which is higher than in previous series reporting a range of 1-4% [22]. This genus has been repeatedly associated with gut health, because of the ability to produce acetate and propionate at the mucus layer, making those SCFAs more available for absorption [23]. In addition, the presence in the mucus layer of some species, such as *A. muciniphila*, could exert anti-inflammatory effects [22]. Other authors highlight its ability

to degrade mucin in the intestinal mucus layer, offering an advantage against other genus when dietary intake of substrates is scarce [24]. *Akkermansia* populations have shown to increase in mice in the presence of some dietary carbohydrates such as fructo-olygosaccharides or some types of resistant starch, or even in the situation of high-fat diets along with pectin or guar-gum [25,26]. Despite *Akkermansia* has been repeatedly found to be reduced in CF, a study showed that the levels after the treatment with a genic therapy reverting the defect in the CFTR protein causing the disease, was able to induce an increase in relative abundance [27]. In this sense, the supplementation with the beta-glucan could be considered to have a similar beneficial effect. In addition, the supplementation induced a significant increase in *Faecalibacterium* abundance in the DC of the CF subject, even when it has been suggested that *in vitro* models are not suitable for some species of this genus [28]. These commensal bacteria are considered as beneficial because of its ability to produce butyrate and its association with the immune system, and could be considered as an anti-inflammatory agent of the gut microbiota [29,30]. Other *in vitro* studies showed increased *Faecalibacterium* in other dysbiotic scenarios, as in the case of CF, when supplementing different types of prebiotics, but not in healthy controls [31]. Similarly, the results of the present study show a decrease in the relative abundance of *Faecalibacterium* in the H subject.

When focusing on the PC of the CF subject, a relevant finding was that *Acidaminococcus*, decreased ($p<0.0001$). This genus has been previously associated with increased values in CF, and its presence in intestinal microbiota has been correlated to lower growth rates in children with this condition [32] and with inflammation indicators such as calprotectin [4,33]. Therefore, the reduction in the abundance of this genus is one of the most relevant findings of the study, and along with increased *Akkermansia* and *Faecalibacterium* and decreased *Pseudomonas*, could support the use of beta-glucan as a

prebiotic for gut dysbiosis correction. The changes in the healthy control, however, suggest that the supplementation with the beta-glucan is not as effective in the microbiota of a healthy subject as in the case of a disrupted microbiota.

Other relevant findings in bacterial genus changes deserving attention are the specific differences related to subject type, including higher *Acidaminococcus* in CF (considered as a marker of dysbiosis in CF [4,33]), and lower *Alistipes* (in accordance with Nielsen et al. (2016) [34]), both in PC and DC, at the three stages of the study. In addition, *Bifidobacterium*, was significantly lower in CF than in H, in the DC, during post-treatment, suggesting that the supplementation with beta-glucan, despite resulted in a healthier microbiota in the CF subject, did not allow for the growth of *Bifidobacterium*. This finding coincides with the study by Nogaka et al. (2020) who described improved microbiota in a dysbiotic *in vitro* environment, but with no changes regarding *Bifidobacterium* [31].

Despite heterogeneity and scarcity of studies on colonic microbiota in CF, it seems well established that the number of species and diversity are lower compared to healthy counterparts [16,35,36]. In fact, while microbiota composition has shown to differ between subjects with CF, lower diversity seems to be one of the few statements that can be made, given the repetition of this finding along the available studies [37]. Another highlight in the results of diversity is that human *in vivo* studies report Shannon indexes in the range of 2-4 [4] while in the in vitro context of our study, these values were 9-10. A consideration in this respect is that *in vitro* models do not take into account several relevant physiological aspects possibly influencing microbiota, such as the intake of compounds other than the prebiotic, gut motility, transit time or environmental exposure.

To our knowledge, only one study has been conducted to address the effect of a prebiotic (high amylose maize starch) on colonic microbiota in CF, and it was in adult

patients and using a static 24-hour colonic *in vitro* fermentation model [9]. The authors found reduced diversity in patients with CF. Other previous studies conducted with the SHIME to study the effect of a prebiotic supplementation in other contexts also reported increased Shannon index and Chao1 values in the distal part of the colon compared to the proximal site [14,38]. The higher pH and the reduction of water content in the last part of the colon tract seem to be favourable conditions for increasing the spectrum of bacterial types that can grow.

Moving onto changes in the metabolic activity, a desirable outcome would be a reduction in ammonia, as high concentrations of this ion has been related to reduce absorption of SCFA [39] and alteration in DNA synthesis, increasing the risk of tumour development [40]. Our results did not show significant changes through study periods, but the baseline levels were already relatively low compared to previous series [41]. In contrast, lactate levels in the PC during the treatment period, in both subjects, were higher compared to previous studies [15]. Lactate exerts a beneficial role by an indirect inhibition of pathogenic bacteria growth [42].

Another relevant microbiota metabolism outcome is the production of SCFA. These metabolites are produced from fermentation of non-digestible carbohydrates, such as beta-glucan, and modulate gut inflammation [23]. The production of SCFA and other metabolites is known to be associated with specific bacterial genera, and our results are coherent with previous studies. For instance, we found that *Bacteroides* had several positive correlations with different SCFA [43], and Veillonella with lactate [34,44]. In accordance with previous studies also, *Faecalibacterium* showed the highest correlation with BA, coinciding with previous findings stating that some species of this genera are the main BA producers in the gut [45]. In addition, *Faecalibacterium* was correlated with other metabolites such as VA, CA, or lactate a, being along with *Bacteroides*, the genus

showing the highest number of positive correlations [5]. Regarding *Akkermansia*, the significant and positive correlation with the production of PA reinforces the previous findings in the literature explaining that this genus produces PA as a result of mucin degradation [23].

Overall, this study presents with the limitation of the *in vitro* environment that prevents from simulating other relevant physiological factors influencing colonic digestion. In addition, this methodology limits the number of subjects that can be included. On the other hand, the methodological approach with the SHIME allowed us to study the CF microbiota modulation with prebiotic compound. and detect CF condition-specific patterns in terms of microbiota change when supplying a prebiotic. The findings in terms of relative abundances, diversity and metabolite production are coherent with previous studies conducted with similar methodologies and design, and the correlations between bacterial genera and metabolites reinforce the validity of the study.

In conclusion, the supplementation with beta-glucan led to different changes in colonic microbiota between CF and H conditions, which were in particular more beneficial for the CF scenario in terms of genera (increased *Faecalibacterium* and *Akkermansia*, decreased *Pseudomonas* and *Acidaminococcus*). These results contribute to enlarge the scarce evidence for the potential of a prebiotic in modifying colonic microbiota in CF, in the *in vitro* environment, and encourage further research in the field of prebiotic supplementation in CF to revert or improve dysbiosis.

Data Availability

All data generated or analysed during this study are available from the corresponding author on reasonable request. The Illumina sequencing raw data was uploaded to the

NCBI database (Submission: ERA23351972, on 15-05-2023) with project reference PRJEB62140, and accession number 326f56c0-a122-4805-94fd-75fc6e881792.

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Author contribution

AAG, AH, JGH, MCC, CRK, AA and JCL conceived and designed research. AAG, AH and JGH conducted experiments. RCR and MCC contributed new reagents or analytical tools. EM and CRK provided the faecal samples and resources. AAG, JCL and RCR analysed data. AAG and JCL wrote the manuscript. All authors read and approved the manuscript.

Competing interest

The authors declare no competing interests.

Consent statement

This study was approved by the Ethics committees of University Hospital La Fe (Valencia, Spain) (Ref. 2021-111-1) and Universitat Politècnica de València (Valencia,

Spain) (Ref. P09_24_11_2021), and perfomerd in accordance with de Declaration of Helinski. Inform consent was obtained from the participating individual's guardian.

FIGURE LEGENDS

Figure 1. Relative abundance of phyla in cystic fibrosis (CF) and healthy (H) microbiota, in both colonic regions (PC: proximal colon and DC: distal colon) in the three periods of the experiment (B: baseline, T: treatment and PT: posttreatment). *: significant differences between periods; a: significant differences between H and CF; A: significant differences between PC and DC.

Figure 2. Relative abundance of genus in cystic fibrosis (CF) and healthy (H) microbiota, in both colonic regions (PC: proximal colon and DC: distal colon) in the three periods of the experiment (B: baseline, T: treatment and PT: posttreatment).

Figure 3. Short chain fatty acids (SCFA) production in cystic fibrosis (CF) and healthy (H) microbiota, in both colonic regions (PC: proximal colon and DC: distal colon) in the three periods of the experiment (B: baseline, T: treatment and PT: posttreatment). AA: acetic acid; PA: propionic acid; BA: butyric acid; VA: valeric acid; CA: caprionic acid; IVA: isovaleric acid; IBA: isobutyric acid. *Significant differences were found in between PC and DC.

Figure 4. Correlation matrix between bacterial genera and metabolites. AA: acetic acid; PA: propionic acid; BA: butyric acid; VA: valeric acid; CA: caprionic acid; IB: isobutyric acid; IVA: isovaleric acid.