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***In vitro* screening of the impact of dietary prebiotic components, probiotic strains, and their symbiotic combinations on colonic microbiota in children with cystic fibrosis†**

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Children with Cystic Fibrosis (CF) are more likely to have intestinal dysbiosis due to recurrent antibiotic therapy and the conventional hypercaloric diet administered to them. This study aimed at evaluating the effect of isolated prebiotic components and probiotic strains, and their combinations as potential symbiotics, on the intestinal microbiota of CF children. A static *in vitro* colonic fermentation model was used by colonizing vials with faecal inoculum, a culture medium, and the substrates to be tested. Post treatment, aliquots were taken to determine ammonium, lactate, and short-chain fatty acids production and to profile the microbiota composition by 16s rRNA sequencing. At genus level, *Escherichia-Shigella* decreased (15.8%) with the treatment pectin + *L. rhamnosus*, followed by the beta-glucan + *L. salivarius* (15.5%). Inversely, the most increase in *Bacteroides* (44%) was obtained by the treatment with Pectin + *L. reuteri*. Lactate and acetic acid production was significantly increased with prebiotics and their combinations with *L. rhamnosus* and *L. salivarius*. In conclusion, the use of beta-glucan and pectin in combination with probiotic strains from the *Lactobacillaceae* family suggest potential to modulate dysbiosis and metabolic activity on CF colonic microbiota, encouraging further studies in animal studies or clinical settings to confirm the findings *in vivo*.

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Introduction

In patients with cystic fibrosis (CF) intestinal dysbiosis is thought to be the consequence of a series of factors, including the abnormal function of the transmembrane conductance regulator protein (CFTR), its mechanisms, and different acquired factors such as repeated exposure to antibiotic therapy and traditional hypercaloric diet.¹ Consequently, the imbalance in the gut microbiota is characterized by increased pathogenic bacteria and reduced microbial diversity, significantly compromising patients' health conditions.² Most of the studies on patients with CF have focused on the lung micro-

biota, so there is scarce scientific evidence about the intestinal microbiota and therapeutic solutions to reverse dysbiosis.³

In this context, there is a need to search for nutritional strategies that help modulating the intestinal microbiota and contribute to improve the prognosis of the disease. A study in children with CF showed that energy requirements are met through diets rich in saturated and trans fats, and poor in fibre, which contribute to gastrointestinal inflammation and microbial dysbiosis, suggesting that the impact of diets with foods rich in fibre, whole grains and resistant starch should be explored, because of the prebiotic potential.⁴ It is well known that both prebiotic and probiotic compounds can contribute to improve gut microbiota and the production of immunomodulatory metabolites in different situations.⁵ Prebiotics can be found naturally present in foods, and they are generally non-digestible compounds that serve as a substrate for host microorganisms and confer health benefits.^{6,7} Non-starch polysaccharides are considered as potential prebiotics. These molecules are carbohydrate fractions excluding starch, mono and disaccharides, differing in composition and structure from amylose and amylopectin. As they cannot be hydrolysed in the upper gastrointestinal tract, these compounds reach the colon where they can be eventually fermented by the gut micro-

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biota.⁸ In addition, resistant starch also escapes gastrointestinal digestion and can be selectively used as substrate for gut microbiota. Different types of resistant starch have been defined,⁹ including retrograded starch, which is formed after a cooling period by gelatinised starch.¹⁰ Up to now, only one *in vivo* study on the use of prebiotics in adults with CF is available.¹¹

On the other hand, probiotics are live microorganisms that when supplied in adequate amounts, induce beneficial effects on the host's health.¹² One of the few *in vivo* studies in CF that evaluated the impact of probiotic supplementation on modifying colonic microbiota as a main study outcome evidenced that *Lactobacillus reuteri* could be effective in reducing some pathogenic bacteria in the gut.¹³ However, other studies on probiotic supplementation suggest contradictory results,¹⁴ due to different methodological limitations and those inherent to the multifactorial nature of CF disease.¹⁵ Likewise, a meta-analysis evaluated the efficacy and safety of probiotics in CF and showed that more research is needed to determine their clinical implications¹⁶

Therefore, an *in vitro* approach to evaluate the potential of different probiotic strains, prebiotic compounds, and synbiotic preparations, in correcting dysbiosis could generate a background of certainty to support the selection of the best “biotics” supplementation strategy. Static *in vitro* colonic fermentation models allow for studying multiple samples and “biotic” substrates simultaneously and evaluating the different effects on intestinal microbiota and metabolic activity.¹⁷ In this way, these models can be considered as a screening tool prior the study of long-term supplementation in dynamic *in vitro* models or in clinical settings.

In fact, *in vitro* colonic fermentation models to assess the effect of potential prebiotic compounds and probiotic strains on modulating gut microbiota in CF have been already applied. These previous studies in the field focused on *Lactobacillaceae* strains and beta-glucan, respectively, both suggesting promising results in improving gut microbiota in the context of CF.^{18,19} However, no information is available on the potential of combining a probiotic strain with a prebiotic compound.

Therefore, this study aimed at evaluating the effect of isolated prebiotic components and probiotic strains, and their combinations as potential synbiotics, on the intestinal microbiota of children with CF, using a static *in vitro* simulation model of the colonic fermentation.

Materials and methods

Subjects

The faecal samples to obtain the faecal inoculum for the colonic fermentation model ($n = 4$) were obtained from paediatric patients with CF recruited in the Valencian Community. The study protocol was approved by the Ethics Committee of Universitat Politècnica de València (P03_25-07-2022). The inclusion criteria were: age between 2 and 16 years old, a diag-

nosis of CF confirmed by a positive sweat test (>60 mEq L⁻¹) and/or by the presence of two CF-causing mutations in the CFTR gene, and a confirmed diagnosis of exocrine pancreatic insufficiency (faecal elastase <200 µg g⁻¹ in stool). The exclusion criteria were the presence of acute infections, acute abdominal pain, treatment with a CFTR gene modulating therapy, the absence of antibiotic treatment in the last 2 months and/or the supplementation with prebiotics or probiotics. All the subjects and their parents/legal guardians signed the informed consent.

Selection of compounds with prebiotic potential and probiotic strains

Dietary fiber types with prebiotic potential were selected following the classification of Fu *et al.* (2022),²⁰ including two types of non-starch polysaccharides: β-glucan (Bgl) from Neogen® (Michigan, USA) and apple pectin (Pc) from Sigma-Aldrich® (Missouri, USA); and two types of resistant starch: native potato starch granules (St) from Sigma-Aldrich® (Missouri, USA) and gelatinized and retrograde starch (rSt) modified by physical processing according to the protocol of Zhou *et al.* (2019).²¹ The selection was made under the criterium of assessing pure compounds (analytical standards) rather than commercially available supplements, as these are formulated with excipients that could interfere with the results.

On the other hand, three probiotic strains from the *Lactobacillaceae* family were selected, based on previous proved beneficial effects in children with CF:^{13,18} *Lacticaseibacillus rhamnosus* GG (ATCC 53103TM) (*L. rha*), *Limosilactobacillus reuteri* (DSM17938) (*L. reu*) and *Lactobacillus salivarius* (CECT 4063) (*L. sal*). *L. rha* and *L. reu* were isolated from probiotic commercial supplements containing only one microorganism strain, Kaleidon Hydro (Menarini®) and Casenbiotic (BioGaia®) respectively. *L. sal* was obtained from the Spanish Collection of Type Cultures (CECT). Then, the strains were grown in MRS liquid medium until obtaining a minimum concentration of 10^8 CFU mL⁻¹.

Materials

Phosphate buffer (0.1M) was obtained from EMD Millipore (Massachusetts, USA). Peptone, sodium chloride, magnesium sulphate, calcium chloride hexahydrate, Tween 80, resazurin salt solution 0.25% (w/v) and bile salts were supplied from Sigma-Aldrich® (Missouri, USA.). Potassium dihydrogen phosphate was purchased from Scharlau® (Barcelona, Spain). Sodium hydrogen carbonate was obtained from Chem-Lab® (Zedelgem, Belgium). Yeast extract was obtained from Condalab® (Madrid, Spain). Hemin, vitamin K1 and cysteine were purchased from Sigma-Aldrich® (Missouri, USA).

Static *in vitro* colonic fermentation

The experiment was conducted using the static *in vitro* colonic fermentation model for foods proposed by Pérez-Burillo *et al.* (2021)²² with some modifications to adapt the simulation to the fermentation of pre and probiotic compounds.^{23,24} In short, fermentation vials were prepared with the faecal inocu-

lum, a culture medium, and the study substrate (prebiotic, probiotic or synbiotic).

The faecal inoculum was prepared from the stool samples of 4 children with CF. All of them had pancreatic insufficiency and their ages were between 6 and 11 years old. None of them had taken antibiotics or supplements in two months before the study, and had not started CFTR modulator therapy. The day of the experiment, the four faecal samples were collected fresh (1–2 hours from deposition) from the house of the donors in the interior of sterile pots with anaerobiosis bags. The samples were transported to the laboratory in refrigeration and immediately processed.

The culture medium contained peptone, sodium chloride, magnesium sulphate, calcium chloride hexahydrate, Tween 80, resazurin salt solution, bile salts, potassium dihydrogen phosphate, sodium hydrogen carbonate, yeast extract were mixed, the mixture was autoclaved and then hemin, vitamin K1 and cysteine were added. Of note, the bile salts concentration was modified by reducing the final concentration to 0.05 g L^{-1} to better approach to the altered concentration found in children with CF.^{25,26} The pH of the medium was adjusted to 6.5 according to the average physiological value reported *in vivo* in CF.²⁷ previously measured before sterilization in the autoclave. The samples were first pooled (1 g each) and mixed with phosphate buffer 0.1 M (1:10 w/v), and the blend was introduced in a stomacher for 2 minutes and the supernatant was collected to inoculate the fermentation vials.

To perform the colonic fermentation of dietary prebiotic components, the concentration of the study substrates was based on a previous study:²⁸ 24 mg of each compound were weighed in sterile vials, to which 5.4 mL of culture medium and 0.6 mL of faecal inoculum were added. For probiotics, 1 mL of each strain (10^8 CFU mL^{-1}), 4.4 mL of culture medium and 0.6 mL of faecal inoculum were mixed. Regarding the synbiotic combinations, 12 different were prepared (3 probiotics \times 4 prebiotics), for which 24 mg of the prebiotic was mixed with 1 mL of resuspended probiotic, 4.4 mL of culture medium and 0.6 mL of faecal inoculum. Additionally, a control vial was prepared (basal microbiota), including 5.4 mL of culture medium and 0.6 mL of faecal inoculum. Finally, oxygen was removed from the vials using a nitrogen gas flow for 30 seconds before sealing, and the vials were introduced into a hermetic chamber, where oxygen was removed with the use of anaerobiosis bags (Thermo Scientific™ Oxoid AnaeroGen). In total, 20 different conditions were tested in triplicate (60 assays were performed). All the samples were incubated in anaerobiosis for 20 h at 37 °C in agitation (20 rpm). After completion of colonic fermentation, different aliquots were taken for subsequent analytical determinations.

Analysis of the colonic microbiota

Microbiota composition by 16S rRNA amplicon gene sequencing. Using the Stool DNA Isolation Kit from Norgen Biotek Corp® (Ontario, Canada), total DNA was extracted from all the samples following manufacturer's instructions. The final yield of the DNA extraction was calculated using fluorometry (a Qubit fluorometer from Invitrogen Co., Carlsbad,

California). V3 and V4 regions of the 16S rRNA gene were PCR amplified by using specific primers. Amplicons were examined using a Bioanalyzer DNA 1000 chip, and libraries were sequenced using a 2×300 bp paired-end run (MiSeq Reagent kit v3) on an MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain).

Sequenced read on Illumina MiSeq platform (2×300 bp) of FISABIO Sequencing Service were submitted to the pipeline of package dada2 (version 1.26.0)²⁹ for R software (R version 4.3.0 (21 April 2023)) for the microbiota data processing. Only R2 reads from Illumina paired ends were truncated at 250 position and reads under 250 nucleotides were removed. Every read with maximum expected error above 2 (expected error calculated from the nominal definition of the quality score ($-\sum 10^{(-Q/10)}$)) was also removed, the same as those which matched against the phiX genome. ASVs (Amplicon Sequence Variants) were inferred from DADA2 algorithm, and chimeras were removed with default parameters. Taxonomic assignment was performed up to genus level, based on SILVA database species train set file (version 138.1). R package phyloseq (version 1.44.0)³⁰ was used for manipulating microbiota data. The alpha diversity (Shannon and Chao indexes) as well as beta diversity (Bray–Curtis scale) were obtained using R software.

Short-chain fatty acids (SCFAs), ammonia and lactate. All the analytical standards for acetic (AA), propionic (PA), butyric (BA), isovaleric (IVA), and isobutyric (IBA) acids were used and provided by Sigma-Aldrich® (Missouri, USA). The protocol was performed as described in,³¹ a preliminary liquid–liquid

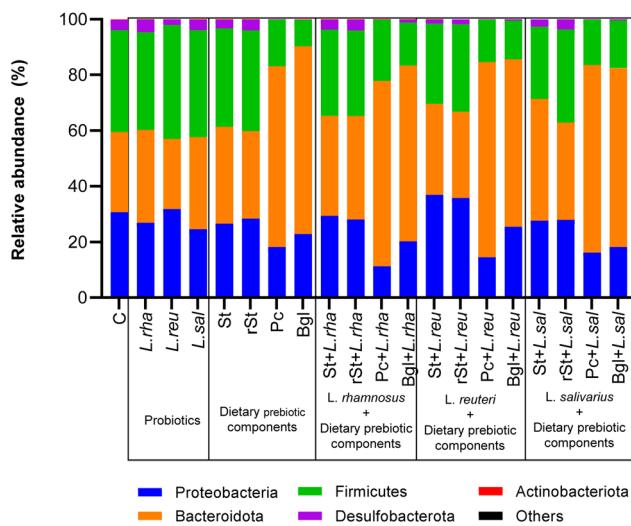


Fig. 1 Microbiota composition at phylum level. Control (C), *L. rhamnosus* (*L. rha*), *L. reuteri* (*L. reu*), *L. salivarius* (*L. sal*), with prebiotics: starch (St); resistant starch (rSt); pectin (Pc); beta-glucan (Bgl); and with synbiotics: starch + *L. rhamnosus* (St + *L. rha*); resistant starch + *L. rhamnosus* (rSt + *L. rha*); pectin + *L. rhamnosus* (Pc + *L. rha*); beta-glucan + *L. rhamnosus* (Bgl + *L. rha*); starch + *L. reuteri* (St + *L. reu*); resistant starch + *L. reuteri* (rSt + *L. reu*); pectin + *L. reuteri* (Pc + *L. reu*); beta-glucan + *L. reuteri* (Bgl + *L. reu*); starch + *L. salivarius* (St + *L. sal*); resistant starch + *L. salivarius* (rSt + *L. sal*); pectin + *L. salivarius* (Pc + *L. sal*); beta-glucan + *L. salivarius* (Bgl + *L. sal*).



extraction with diethylether applied where the aliquots of the fermented samples (2 mL) were homogenized with 0.5 mL of sulphuric acid (9.2M), and a small amount of sodium chloride was added to remove any lingering traces of water. Following that, 0.4 mL of the internal standard solution (2-methylhexanoic acid (52.9 mM)) after the addition of 2 mL of diethylether, the mixture was vortexed for 1 minute and centrifuged at 3000 rpm for 3 min. SCFA from the samples was analysed by gas chromatography with flame ionization (GC-FID). GC-FID analysis was carried out on the Agilent GC7890B-5977B gas chromatograph by Agilent Technologies® (La Jolla, EE.UU.), apparatus with a multipurpose sampler and a HP-INNOWAX™ C30 capillary GC column (30 m × 0.25 mm × 0.25 µm). The injector temperature was set at 250 °C. Results were expressed in micromolar concentration (µM).

Following the guidelines and recommendations of the manufacturer, the lactate concentration was measured using the Lactate Assay commercial enzyme kit from Sigma-Aldrich® (Missouri, USA) and the ammonia concentration was measured using the Ammonia commercial enzyme kit from R-Biopharm® (Darmstadt, Germany). Results were expressed in micromolar concentration (µM).

Statistical analyses

The statistical analysis to study the metabolic activity of the colonic microbiota was performed with the GraphPad Software® (Massachusetts, USA) version 8.4.3. This analysis included the execution of a Dunnett test for multiple comparisons (two-way ANOVA) (alpha level 0.05) using three replicates of basal microbiota samples, as well as three real replicates of each compound to assess possible significant differences. Pearson correlations were applied to assess the possible relations between metabolites and the microbial genera.

Results

Response of colonic microbiota to dietary prebiotics, probiotics and synbiotics

The basal microbiota of the pooled inoculum was defined by alpha diversity with Chao index of 6.8 and Shannon index of 2938. The treatments induced changes in the microbiota composition after simulated the colonic fermentation. Focusing on beta diversity (ESI Fig. 1†), the treatments that resulted in a most different diversity compared to the control, as expressed by Bray-

Table 1 Statistically significant differences (Two-way ANOVA) in faecal microbiota bacterial phylum after colonic fermentation with the different treatments compared to basal microbiota

Phylum	Treatment	Relative abundance (%)	P value	Adjusted P value
Firmicutes	Basal microbiota	36.60		
	Pectin	16.77	<0.0001	<0.0001
	Beta-glucan	9.44	<0.0001	<0.0001
	Starch with <i>L. rhamnosus</i>	30.91	0.0024	0.0315
	Resistant starch with <i>L. rhamnosus</i>	30.65	0.0016	0.0214
	Pectin with <i>L. rhamnosus</i>	21.88	<0.0001	<0.0001
	Beta-glucan with <i>L. rhamnosus</i>	15.51	<0.0001	<0.0001
	Starch with <i>L. reuteri</i>	28.68	<0.0001	0.0007
	Pectin with <i>L. reuteri</i>	15.25	<0.0001	<0.0001
	Beta-glucan with <i>L. reuteri</i>	13.90	<0.0001	<0.0001
Proteobacteria	Starch with <i>L. salivarius</i>	25.89	<0.0001	<0.0001
	Pectin with <i>L. salivarius</i>	16.39	<0.0001	<0.0001
	Beta-glucan with <i>L. salivarius</i>	17.17	<0.0001	<0.0001
	Basal microbiota	30.75		
	<i>L. salivarius</i>	24.60	0.0011	0.0157
	Pectin	18.23	<0.0001	<0.0001
	Beta-glucan	22.89	<0.0001	0.0008
	Pectin with <i>L. rhamnosus</i>	11.33	<0.0001	<0.0001
	Beta-glucan with <i>L. rhamnosus</i>	20.31	<0.0001	<0.0001
	Starch with <i>L. reuteri</i>	36.87	0.0012	0.0164
Bacteroidota	Pectin with <i>L. reuteri</i>	14.56	<0.0001	<0.0001
	Pectin with <i>L. salivarius</i>	16.18	<0.0001	<0.0001
	Beta-glucan with <i>L. salivarius</i>	18.18	<0.0001	<0.0001
	Basal microbiota	28.79		
	Pectin	64.88	<0.0001	<0.0001
	Beta-glucan	67.37	<0.0001	<0.0001
	Starch with <i>L. rhamnosus</i>	35.97	0.0002	0.0028
	Resistant starch with <i>L. rhamnosus</i>	37.18	<0.0001	0.0003
	Pectin with <i>L. rhamnosus</i>	66.62	<0.0001	<0.0001
	Beta-glucan with <i>L. rhamnosus</i>	63.09	<0.0001	<0.0001



Courtis scale, were the symbiotic combinations of pectin and beta glucan with all the prebiotics. In addition, the changes in microbiota composition were more in detail assessed by comparing the microbial abundances at different taxonomic levels.

At phylum level, the basal microbiota was mainly characterised by the presence of Firmicutes (36.6% of relative abundance), followed by Proteobacteria (30.75%), Bacteroidota (28.79%), Desulfobacterota (3.67%) and Actinobacteriota (0.12%). The impact of static colonic fermentation of samples with probiotic strains, dietary prebiotic components, and their combinations on the composition of microbiota, are presented in Fig. 1. Statistically significant differences at phylum level were found between the basal microbiota *versus* the treatments (Table 1). The relative abundance of Firmicutes and Proteobacteria in the basal microbiota was significantly different from the most treatments to different extents and exceptions. Beta-glucan showed the highest reduction of Firmicutes (~27%), and the most reduction in Proteobacteria (~19%) was achieved by the treatment with Pectin + *L. rhamnosus*. Inversely, Bacteroidota was increased with pectin and beta-glucan and the symbiotics. Pectin + *L. reuteri* represented the highest increased of Bacteroidota (+41%).

Going into the genus level, *Acidaminococcus* represented the highest relative abundance of the basal microbiota (27.46%), followed by *Escherichia-Shigella* (26.29%), *Bacteroides* (22.21%), *Proteus* (3.25%), *Bilophila* (2.23%), and *Alistipes* (1.47%) (Fig. 2). Some treatments were able to modify the proportion of the different genera of the microbiota.

Statistically significant differences at genus level between the basal microbiota *versus* dietary prebiotic components, probiotic strains and their combinations were found (Table 2). Some treatments changed the *Acidaminococcus* and *Escherichia-Shigella* ratio, beta-glucan alone being the one that reduced the most the relative abundance of *Acidaminococcus* by 23%. In the case of *Escherichia-Shigella*, pectin + *L. rhamnosus* was able to impart 15.8% decrease, followed by the beta-glucan + *L. salivarius* treatment (~15.5%). Inversely, the highest increase in *Bacteroides* (+44%) was obtained with the treatment with Pectin + *L. reuteri*.

Metabolite production during static colonic fermentation of dietary prebiotic components, probiotic strains, and their combination

The dietary prebiotic components, probiotic strains, and their combinations influenced ammonia and lactate production (Fig. 3) (ESI Table 1†). Ammonia concentration showed a significant decrease with two dietary prebiotic components: pectin 1826.38 $\mu\text{mol L}^{-1}$ (244.05) (adjusted $p < 0.0001$) and beta-glucan 1951.02 $\mu\text{mol L}^{-1}$ (386.42) (adjusted $p < 0.0001$) compared to basal microbiota 3703.10 $\mu\text{mol L}^{-1}$ (84.74) (Fig. 3a). Similarly, lactate production was significantly increased with *L. rhamnosus* alone and with their combinations with prebiotics. The same increased was observed with treatments combining *L. salivarius* with prebiotics as referred to the basal microbiota. No statistically significant differences were found with the treatments with *L. reuteri* (Fig. 3b).

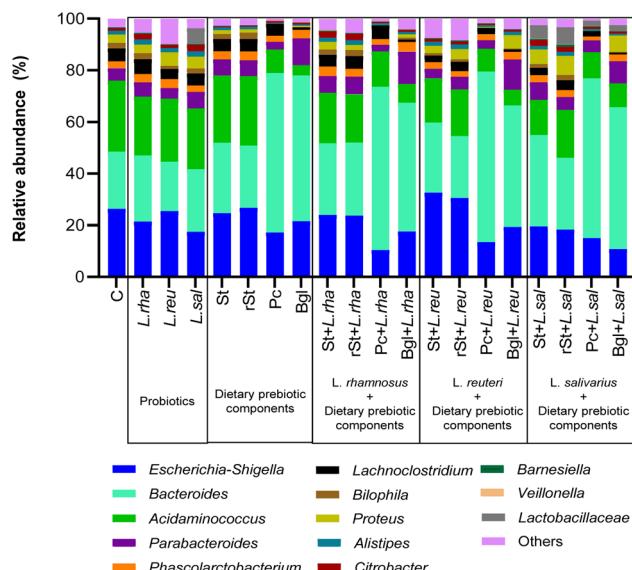


Fig. 2 Microbiota composition at genus level. Control (C), *L. rhamnosus* (*L. rha*), *L. reuteri* (*L. reu*), *L. salivarius* (*L. sal*), with prebiotics: starch (St), resistant starch (rSt), pectin (Pc), beta-glucan (Bgl), and with symbiotics: starch + *L. rhamnosus* (St + *L. rha*), resistant starch + *L. rhamnosus* (rSt + *L. rha*), pectin + *L. rhamnosus* (Pc + *L. rha*), beta-glucan + *L. rhamnosus* (Bgl + *L. rha*); starch + *L. reuteri* (St + *L. reu*), resistant starch + *L. reuteri* (rSt + *L. reu*), pectin + *L. reuteri* (Pc + *L. reu*), beta-glucan + *L. reuteri* (Bgl + *L. reu*); starch + *L. salivarius* (St + *L. sal*), resistant starch + *L. salivarius* (rSt + *L. sal*), pectin + *L. salivarius* (Pc + *L. sal*), beta-glucan + *L. salivarius* (Bgl + *L. sal*).

Probiotics alone, and prebiotics alone did not significantly alter the concentrations of propionic acid, butyric acid and BCFAs, with only a reduction in acetic acid obtained with pectin and beta-glucans alone (Fig. 4). Comparably, no changes occurred with the combined treatments of starch and resistant starch with probiotics, but the combinations with pectin and beta-glucan led to significant increases of acetic acid and propionic acid (Fig. 4a and b). No statistically significant differences in butyric acid and BCFAs concentrations were found with respect to the basal microbiota (Fig. 4c and d).

Correlations between genera and metabolites

Statistically significant correlations were found between some bacterial genera and metabolite production (Fig. 5). *Escherichia-Shigella* was found in a positive correlation with lactate. *Bacteroides* showed positive correlations with AA and PA. *Acidaminococcus* was negatively associated with PA and BA. *Bilophila* showed negative associations with AA, PA, and BA, while *Proteus* was positively associated with IBA and, *Alistipes* had a negative correlation with PA.

Discussion

This study evaluated the effect of 3 probiotic strains, 4 dietary prebiotic components, and their 12 combinations in terms of changes in the faecal microbiota of children with CF after



Table 2 Statistically significant differences (Two-way ANOVA) in faecal microbiota bacterial genus after colonic fermentation with the different treatments compared to basal microbiota

Genus	Treatment	Relative abundance (%)	Pvalue	Adjusted P value
<i>Acidaminococcus</i>	Basal microbiota	27.46		
	Pectin	9.13	<0.0001	<0.0001
	Beta-glucan	4.03	<0.0001	<0.0001
	Starch with <i>L. rhamnosus</i>	19.61	<0.0001	<0.0001
	Resistant starch with <i>L. rhamnosus</i>	18.76	<0.0001	<0.0001
	Pectin with <i>L. rhamnosus</i>	13.56	<0.0001	<0.0001
	Beta-glucan with <i>L. rhamnosus</i>	7.31	<0.0001	<0.0001
	Starch with <i>L. reuteri</i>	17.05	<0.0001	<0.0001
	Resistant starch with <i>L. reuteri</i>	18.09	<0.0001	<0.0001
	Pectin with <i>L. reuteri</i>	8.84	<0.0001	<0.0001
	Beta-glucan with <i>L. reuteri</i>	6.09	<0.0001	<0.0001
	Starch with <i>L. salivarius</i>	13.65	<0.0001	<0.0001
	Resistant starch with <i>L. salivarius</i>	18.62	<0.0001	<0.0001
<i>Escherichia-Shigella</i>	Pectin with <i>L. salivarius</i>	10.06	<0.0001	<0.0001
	Beta-glucan with <i>L. salivarius</i>	9.21	<0.0001	<0.0001
	Basal microbiota	26.29		
	<i>L. rhamnosus</i>	21.35	0.0024	0.0319
	<i>L. salivarius</i>	17.50	<0.0001	<0.0001
	Pectin	17.32	<0.0001	<0.0001
	Pectin with <i>L. rhamnosus</i>	10.49	<0.0001	<0.0001
	Beta-glucan with <i>L. rhamnosus</i>	17.74	<0.0001	<0.0001
	Starch with <i>L. reuteri</i>	32.59	0.0001	0.0023
	Pectin with <i>L. reuteri</i>	13.47	<0.0001	<0.0001
	Beta-glucan with <i>L. reuteri</i>	19.38	<0.0001	0.0006
	Starch with <i>L. salivarius</i>	19.51	<0.0001	0.0008
	Resistant starch with <i>L. salivarius</i>	18.40	<0.0001	<0.0001
<i>Bacteroides</i>	Pectin with <i>L. salivarius</i>	15.05	<0.0001	<0.0001
	Beta-glucan with <i>L. salivarius</i>	10.75	<0.0001	<0.0001
	Basal microbiota	22.21		
	Starch	27.36	0.0012	0.0169
	Pectin	61.61	<0.0001	<0.0001
	Beta-glucan	56.37	<0.0001	<0.0001
	Starch with <i>L. rhamnosus</i>	27.81	0.0006	0.0094
	Resistant starch with <i>L. rhamnosus</i>	28.34	0.0002	0.0032
	Pectin with <i>L. rhamnosus</i>	63.22	<0.0001	<0.0001
	Beta-glucan with <i>L. rhamnosus</i>	49.76	<0.0001	<0.0001
	Starch with <i>L. reuteri</i>	27.20	0.0022	0.0290
	Pectin with <i>L. reuteri</i>	65.98	<0.0001	<0.0001
	Beta-glucan with <i>L. reuteri</i>	47.04	<0.0006	<0.0001
	Starch with <i>L. salivarius</i>	35.44	<0.0001	<0.0001
	Resistant starch with <i>L. salivarius</i>	27.69	<0.0001	0.0119
	Pectin with <i>L. salivarius</i>	61.86	<0.0001	<0.0001
	Beta-glucan with <i>L. salivarius</i>	55.01	<0.0001	<0.0001

colonic fermentation. The results showed that the combination of beta-glucan and pectin with any of the three probiotic strains led to the most beneficial effects both on colonic microbiota populations and metabolite production, compared to the rest of the combinations and the use of the prebiotics and probiotics alone. Complementary the assessment of beta diversity supported these observations.

The first result to comment is the representativity of the pooled faecal sample (basal microbiota) of the composition and diversity of that in children with CF. Both Chao and Shannon indexes were comparable to a previous study on the *in vitro* simulation of colonic fermentation in CF, and lower than in the microbiota of healthy controls.¹⁹

In terms of bacterial composition, the basal microbiota was found to be reduced in Bacteroidota and increased in Firmicutes compared to previous series of healthy subjects, coinciding with the literature on altered microbiota in

CF.^{2,15,32} Our study demonstrated that pectin and beta-glucan alone and in combination with the three probiotic strains (*L. rhamnosus*, *L. reuteri*, and *L. salivarius*) were effective in reducing the relative abundance of Firmicutes, which was the predominant phylum in the basal microbiota, and significantly increased Bacteroidota. The increase in Bacteroidota may be relevant as the predominance of this phylum in the gut environment would prevent from the growth and permanence of the other phyla competing for the same niche.³³ In addition, species within Bacteroidota possess carbohydrate-active enzymes that degrade undigested carbohydrates into SCFA, which can be used as an additional source of energy, even for the host, after absorption in the enterocytes.³⁴ This would be especially relevant in the case of children with CF, in which part of the dietary macronutrients are not adequately digested or absorbed, implying significant loss of energy uptake.³⁵ On the other hand, the combinations of pectin with



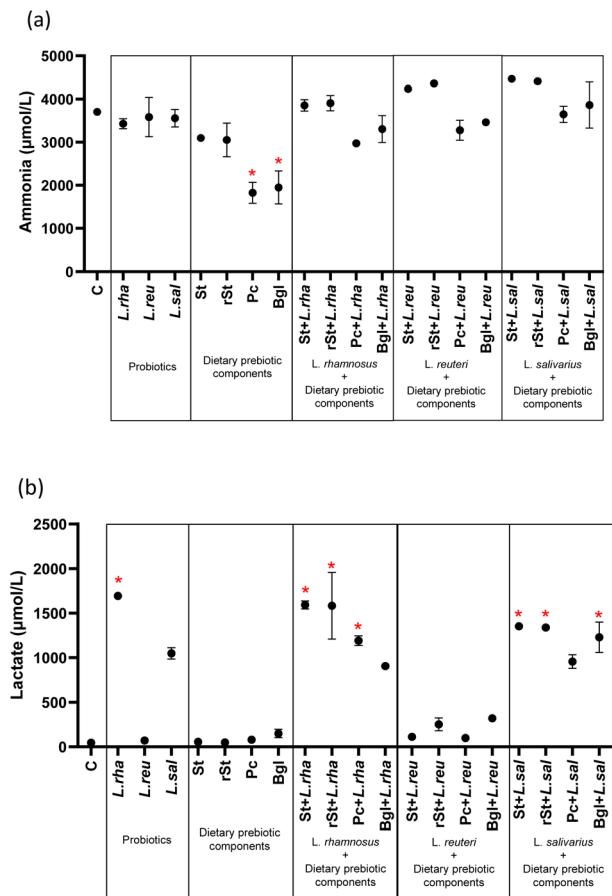


Fig. 3 Metabolite production by colonic microbiota during static *in vitro* simulation of colonic fermentation: (a) ammonia (b) lactate- * statistically significant difference. Control (C), *L. rhamnosus* (*L. rha*), *L. reuteri* (*L. reu*), *L. salivarius* (*L. sal*), with prebiotics: starch (St), resistant starch (rSt), pectin (Pc), beta-glucan (Bgl), and with synbiotics: starch + *L. rhamnosus* (St + *L. rha*), resistant starch + *L. rhamnosus* (rSt + *L. rha*), pectin + *L. rhamnosus* (Pc + *L. rha*), beta-glucan + *L. rhamnosus* (Bgl + *L. rha*); starch + *L. reuteri* (St + *L. reu*), resistant starch + *L. reuteri* (rSt + *L. reu*), pectin + *L. reuteri* (Pc + *L. reu*), beta-glucan + *L. reuteri* (Bgl + *L. reu*); starch + *L. salivarius* (St + *L. sal*), resistant starch + *L. salivarius* (rSt + *L. sal*), pectin + *L. salivarius* (Pc + *L. sal*), beta-glucan + *L. salivarius* (Bgl + *L. sal*). The data shown are mean values from independent triplicates and the standard deviation.

the three probiotic strains were able to reduce to a greater extent the Proteobacteria phylum, which is associated with pathogenic bacteria that cause intestinal inflammation.³⁶ Therefore, these treatments could be considered effective in improving dysbiosis in the microbiota of children with CF.

With respect to the other prebiotics, beta-glucan as well as pectin alone and combined with *L. reuteri* significantly reduced *Acidaminococcus*. This opportunistic pathogen is positively associated with gastrointestinal cancer genes,³⁷ and related to increased calprotectin levels (a marker of intestinal inflammation) in patients with CF.³⁸ Likewise, pectin together with the three probiotic strains under study and beta-glucan with *L. salivarius* were significantly associated with the decrease in the relative abundance of *Escherichia-Shigella*; this

taxon is characteristic in CF and is linked to intestinal dysbiosis.³⁹ In turn, the combinations of pectin and the three probiotic strains, induced a significant increase in *Bacteroides*, which is considered a positive finding, as this genus is involved in the modulation of the immune system.⁴⁰

Focusing on the production of metabolites, significant changes were found in ammonia production, which is a by-product of protein fermentation, and has been associated with negative effects on the organism, such as decreased catabolism of SCFAs and inhibition of mitochondrial oxygen consumption.^{41,42} Concretely, it was reduced by 50.7% and 47.3% of the concentration of the basal microbiota in the presence of pectin and beta-glucan, respectively. This suggests that the two dietary prebiotics act efficiently in the reduction of ammonia, because they stimulate carbohydrate-fermenting bacteria, which increase the colon's acidity and reduce the capacity of protein-fermenting bacteria.⁴³ The reduction of protein-fermenting bacteria would be of special interest in the gut of children with CF, as the presence of protein in the colon is supposed to be increased as a consequence of maldigestion and malabsorption of this nutrient during the small intestine stage.³⁵ Similar results on ammonia were reported in patients with liver cirrhosis supplemented with another prebiotic, xylooligosaccharide (XOS).⁴⁴ In turn, the highest lactate production, was found in the samples treated with *L. rhamnosus* which coincides with a previous study of our group, in which *L. rhamnosus* supplementation reflected the increase in lactate after 20 days of supplementation on colonic microbiota of children with CF.¹⁸ This finding is relevant since it is known that lactate is a beneficial metabolite that exerts a positive role on the body such as regulating the biological processes of intestinal function, produces an indirect inhibition of the growth of pathogenic bacteria, and the genus *Lactobacillus* is related to its biosynthesis to produce propionate, butyrate, or acetate.^{45,46}

In the case of the production of acetic acid and propionic acid, the combination of pectin with *L. rhamnosus* allowed for a two-fold increase of the amount of these metabolites with respect to the initial content. Besides, SCFAs and the genus *Bacteroides* showed a positive correlation, suggesting a symbiotic effect of pectin-*L. rhamnosus*: the changes induced in the microbiota, such as the increase in *Bacteroides*, seem to modify the metabolism of pectin, resulting in higher short-chain fatty acids production. Overall, higher levels of SCFA contribute to improve the immune system, among other beneficial health effects.⁴⁷⁻⁴⁹

The relevance of the study is that new evidence on the role of different probiotics, prebiotics and their symbiotic combinations on CF gut dysbiosis has been generated, in an emerging study field where scarce or null knowledge is available.^{50,51} The new findings are to be interpreted with caution as the study was carried out in an *in vitro* setting. Besides, we acknowledge the limitation of the colonic fermentation model, which despite being adapted to the CF intestinal conditions, might not be fully representative of a CF colon. However, the results can help guiding the focus on which pre-, pro- and synbiotics could be targeted in more complex studies in the future. This aligns with the current context in which the advance in the therapies for CF have



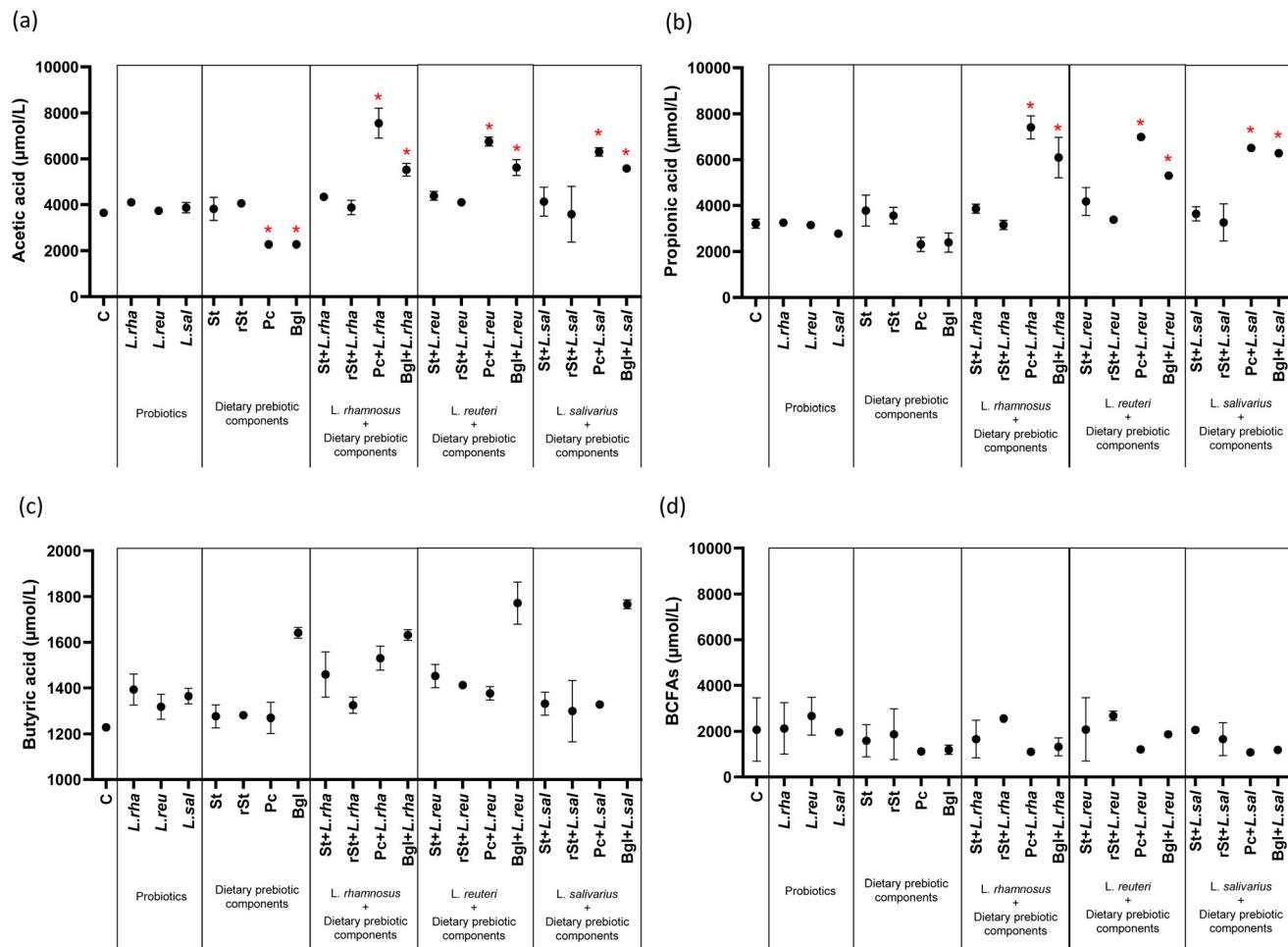


Fig. 4 Metabolite production during static *in vitro* simulation of colonic fermentation: (a) acetic acid, (b) propionic acid, (c) butyric acid, (d) sum of branched chain fatty acids, BCFAs, including isovaleric acid and isobutyric acids. * Statistically significant differences. Control (C), *L. rhamnosus* (*L. rha*), *L. reuteri* (*L. reu*), *L. salivarius* (*L. sal*), with prebiotics: starch (St), resistant starch (rSt), pectin (Pc), beta-glucan (Bgl), and with synbiotics: starch + *L. rhamnosus* (St + *L. rha*), resistant starch + *L. rhamnosus* (rSt + *L. rha*), pectin + *L. rhamnosus* (Pc + *L. rha*), beta-glucan + *L. rhamnosus* (Bgl + *L. rha*); starch + *L. reuteri* (St + *L. reu*), resistant starch + *L. reuteri* (rSt + *L. reu*), pectin + *L. reuteri* (Pc + *L. reu*), beta-glucan + *L. reuteri* (Bgl + *L. reu*); starch + *L. salivarius* (St + *L. sal*), resistant starch + *L. salivarius* (rSt + *L. sal*), pectin + *L. salivarius* (Pc + *L. sal*), beta-glucan + *L. salivarius* (Bgl + *L. sal*). The data shown are mean values from independent triplicates and the standard deviation.

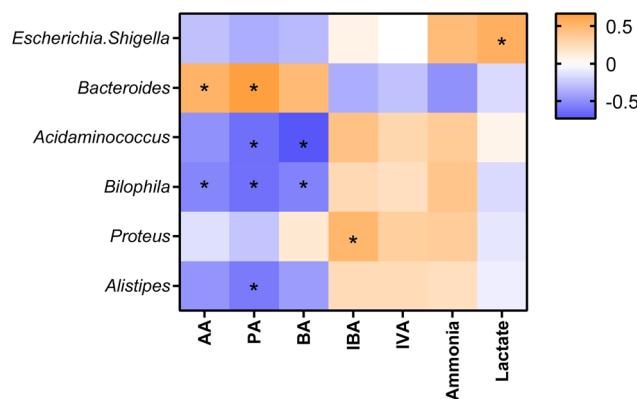


Fig. 5 Pearson correlation coefficients between metabolites and bacterial genera.

led to higher quality of life and better disease prognosis and survival. Therefore, other challenges can be addressed, such as the assessment of dietary interventions as a strategy to improve nutritional status and gut microbiota, including the supplementation with pre-, pro- and synbiotics.

In conclusion, the use of beta-glucan and pectin in combination with probiotic strains from the *Lactobacillaceae* family are suggested as effective approaches to revert modulate dysbiosis and metabolic activity in colonic microbiota of children with CF, future animal studies or clinical settings are encouraged to confirm the findings *in vivo*.

Author contributions

Jazmín Viteri-Echeverría: Methodology, validation, formal analysis, investigation, writing – original draft. Ana Andrés:

Conceptualization, resources, writing – review & editing, project administration. Joaquim Calvo-Lerma: Conceptualization, writing – original draft, writing – review & editing. Ana Heredia: Writing – review & editing. Jorge García-Hernández: Writing – review & editing. Andrea Asensio-Grau: Conceptualization, resources, formal analysis, writing – review & editing, supervision, project administration.

Conflicts of interest

There are no conflicts to declare.

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