

Opportunistic bacteria confer the ability to ferment prebiotic starch in the adult cystic fibrosis gut

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ABSTRACT

Chronic disruption of the intestinal microbiota in adult cystic fibrosis (CF) patients is associated with local and systemic inflammation, and has been linked to the risk of serious comorbidities. Supplementation with high amylose maize starch (HAMS) might provide clinical benefit by promoting commensal bacteria and the biosynthesis of immunomodulatory metabolites. However, whether the disrupted CF gut microbiota has the capacity to utilise these substrates is not known. We combined metagenomic sequencing, *in vitro* fermentation, amplicon sequencing, and metabolomics to define the characteristics of the faecal microbiota in adult CF patients and assess HAMS fermentation capacity. Compared to healthy controls, the faecal metagenome of adult CF patients had reduced bacterial diversity and prevalence of commensal fermentative clades. *In vitro* fermentation models seeded with CF faecal slurries exhibited reduced acetate levels compared to healthy control reactions, but comparable levels of butyrate and propionate. While the commensal genus *Faecalibacterium* was strongly associated with short chain fatty acid (SCFA) production by healthy microbiota, it was displaced in this role by *Clostridium sensu stricto* 1 in the microbiota of CF patients. A subset of CF reactions exhibited enterococcal overgrowth, resulting in lactate accumulation and reduced SCFA biosynthesis. The addition of healthy microbiota to CF faecal slurries failed to displace predominant CF taxa, or substantially influence metabolite biosynthesis. Despite significant microbiota disruption, the adult CF gut microbiota retains the capacity to exploit HAMS. Our findings highlight the potential for taxa associated with the altered CF gut microbiota to mediate prebiotic effects in microbial systems subject to ongoing perturbation, irrespective of the depletion of common commensal clades.

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Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder that results in altered transport of electrolytes and fluid across epithelial surfaces. Chronic suppurative respiratory disease, arising due to impaired clearance of dehydrated airway secretions, is typically the principal cause of morbidity and mortality.¹ However, CF is also associated with chronic gastrointestinal (GI) disease.^{2–4} Impaired mucin hydration, reduced bicarbonate secretion, abnormal bile salt physiology, and impaired fat absorption, all contribute to GI dysfunction and chronic inflammation.^{2,5} In turn, GI inflammation is implicated in mucosal abnormalities and increased intestinal permeability²,

and contributes to an increased risk of a range of intestinal morbidities, including cancer.⁶ Indeed, rates of GI malignancy are significantly higher in patients with CF compared with the general population.^{7–9} Chronic local inflammation also contributes to a systemic pro-inflammatory state, with GI inflammation linked to elevated circulating cytokines and chemokines, soluble adhesion molecules, and acute phase reactants.¹⁰

The abnormal CF gut environment caused by intestinal exocrine malfunction, combined with the effects of antibiotic therapies for chronic lung infections, results in significant alteration of the gut microbiota.^{11–18} As a consequence, CF patients

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exhibit a reduced relative abundance of commensal fermentative bacteria, including members of the *Bifidobacterium* genus^{11,12,15,16} and *Clostridium* clusters XIVa and IV (which include *Faecalibacterium prausnitzii* and *Ruminococcus bromii*).^{11,15-17} At the same time, the relative abundance of pro-inflammatory or potentially pathogenic taxa, particularly Proteobacteria (including *Escherichia coli*) and enterococci (including *Enterococcus faecalis* and *Enterococcus faecium*) is increased.^{13,14,16,17}

Altered CF gut microbiology is likely to compound the pro-inflammatory effects of the underlying disease. In non-CF contexts, alterations to the intestinal microbiota, sometimes referred to as “dysbiosis”, has been shown to contribute to the dysregulation of mucosal immunity¹⁹, and an increase in levels of genotoxic, pro-inflammatory, and hepatomodulatory metabolites.²⁰ By preventing carbohydrate fermentation by members of the colonic microbiota, changes in microbiota composition can also result in reduced production of protective metabolites, including short chain fatty acids (SCFAs).^{21,22} SCFAs have a wide range of anti-inflammatory properties, including the ability to increase colonic regulatory T cells²³, alter dendritic cell and macrophage function²⁴, and reduce production of pro-inflammatory cytokines.^{25,26} SCFAs are also important in the regulation of glycaemic responses and triglyceride metabolism²⁷, are essential for colonic epithelial homeostasis²⁸, and barrier function²⁹, and contribute substantially to energy reclamation from the gut.³⁰ Reduced SCFA production might therefore be critical in CF, where patients experience chronic inflammation and nutritional malabsorption.

Prebiotics are non-digestible compounds that confer health benefits by promoting the proliferation of commensal gut species or the production of beneficial metabolites. These compounds include resistant starches (RS); complex carbohydrates that are not digested and absorbed in the small intestine, but pass into the large bowel and act as substrates for bacterial fermentation.³¹ By increasing the levels of fermentation substrate in the colon, supplementation with compounds such as high amylose maize starch (HAMS) can increase both the abundance of potentially beneficial microbes³², and the production of SCFAs.³³ RS

supplementation has proven to be effective in reducing the risk of both cancer and diabetes by suppressing the development of epithelial adducts³⁴, and reducing insulin resistance and markers of systemic inflammation.³⁵

However, despite a growing interest in the potential clinical benefits of dietary prebiotics in CF, it is not known whether the CF intestinal microbiota retains the capacity to exploit these substrates. Clinical prebiotic use typically assumes the competence of the recipient’s microbiome in utilising the supplemented substrates. A failure to assess microbial capacity prior to supplementation potentially contributes to inter-subject differences in prebiotic response.³⁶ Prebiotic approaches also typically fail to consider the potential to cause detrimental changes in individuals in whom the gut microbiota is already substantially disrupted, such as the selective promotion of potentially pathogenic taxa.

Our aim was to investigate the potential for HAMS supplementation to provide microbiome-mediated benefit in patients with CF. We undertook a detailed metagenomic analysis of the faecal microbiome composition and functional capacity in adult CF patients. Utilising an *in vitro* fermentation model, we then assessed the response to HAMS of faecal microbiota from CF patients and healthy non-CF controls, including the resulting changes in microbiota structure, global metabolomic output, and SCFA production.

Results

Faecal microbiota characteristics in adults with CF

Faecal microbiota composition differed significantly between CF patients and healthy non-CF controls (Figure 1A-B). Taxa richness was reduced in CF samples (median observed OTUs, CF: 272 [IQR: 107], control: 496 [IQR: 102], $p < 0.0001$), as was Faith’s phylogenetic diversity (median diversity, CF: 10.1 [IQR: 3.3]; control: 16.4 [IQR: 3.7], $p < 0.0001$). Simpson’s evenness did not differ significantly between groups ($p = 0.27$). Beta diversity, assessed based on weighted UniFrac distances, also differed significantly between CF samples and controls ($P(\text{perm}) = 0.0001$, square root estimated

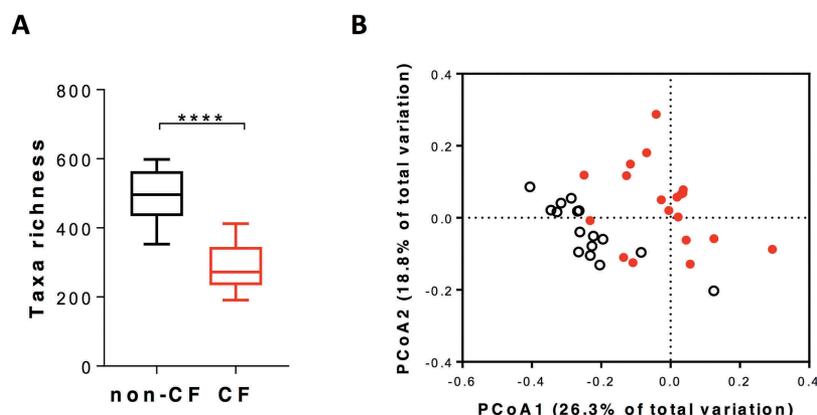


Figure 1. Microbiota characteristics of CF (red) and non-CF (black) faecal samples at baseline (pre-fermentation). **A**) Observed OTUs between microbiota community of CF and non-CF samples. **B**) Principal coordinate analysis (PCoA) derived from weighted UniFrac distance of CF and non-CF samples, PERMANOVA, $P(\text{perm}) = 0.0001$, Pseudo-F = 7.27.

components of variation (ECV) = 0.19, 9,919 permutations; **Figure 1B**). Differences in microbiota composition were characterised by a significantly greater relative abundance of important commensal taxa, such as *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Prevotella copri*, and *Ruminococcus bromii* in non-CF subjects, and a greater relative abundance of *Clostridium symbiosum*, *Veillonella parvula*, *Enterococcus faecalis*, and *Ruminococcus gnavus* in patients with CF (alpha < 0.05 for Kruskal-Wallis test and LDA > 4, data not shown).

The representation of functional genes within both CF and healthy faecal metagenomes also differed significantly ($P(\text{perm}) = 0.0019$, ECV = 6.15, 9,939 permutations). This divergence included significant differences in the proportion of normalised reads that mapped to individual KEGG pathways, including important genes involved in carbohydrate metabolism (Supplementary Figure 1). The gene encoding phosphotransacetylase (*pta*), an enzyme that catalyses the conversion of acetyl-CoA to acetyl-phosphate in the acetate biosynthesis pathway, was significantly lower in CF metagenomes compared to healthy controls (Supplementary Figure 2A). In contrast, *galA*, the gene that encodes alpha-galactosidase in the galactose metabolic pathway, was significantly more highly represented in CF metagenomes (Supplementary Figure 1). No significant differences were observed in the representation of normalised levels of butyrate or propionate biosynthesis pathway genes in CF and healthy controls (Supplementary Figure 2B-C), suggesting that the

fermentation pathways resulting in the production of these SCFAs is conserved in the CF metagenome.

SCFA production during *in vitro* fermentation of HAMS

Levels of acetate, butyrate, propionate, and total SCFA were determined before and after *in vitro* HAMS fermentation by CF and control faecal slurries (**Figure 2A-D**). Acetate levels were significantly lower in CF reactions compared to controls (median concentration, CF: 9.36 mmol/L [IQR: 6.63], control: 21.48 mmol/L [IQR: 11.40], $p = 0.0007$, **Figure 2A**). While total SCFA levels were also significantly reduced (median concentration, CF: 15.03 mmol/L [IQR: 12.77], control: 28.74 mmol/L [IQR: 5.89], $p = 0.0001$, **Figure 2D**), levels of butyrate and propionate did not differ significantly between groups.

SCFA production was associated with changes in the relative abundance of different bacterial taxa in CF and control reactions following HAMS fermentation (**Figure 3A-F**; Supplementary Figure 3). In healthy controls, acetate concentration showed a strong positive correlation with the relative abundance of *Dorea* ($r = 0.78$, $p = 0.021$, **Figure 3A**) and *Anaerostipes* ($r = 0.72$, $p = 0.039$, Supplementary Figure 3); butyrate concentrations were positively correlated with relative abundance of the *Faecalibacterium* ($r = 0.84$, $p = 0.007$, **Figure 3B**); and propionate concentration was significantly correlated with the relative abundance of the *Bacteroides* ($r = 0.77$, $p = 0.049$, **Figure 3C**). In reactions using CF faecal inocula, butyrate levels were strongly correlated with the relative abundance of

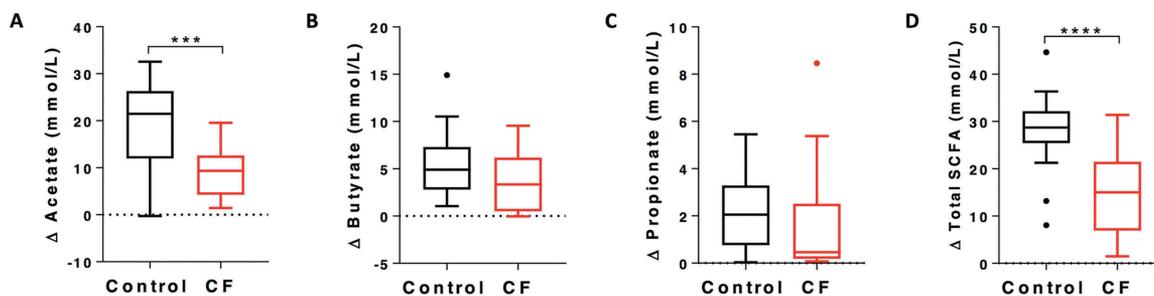


Figure 2. SCFA production of non-CF control and CF samples during the *in vitro* fermentation. Net production (post-fermentation subtracted from pre-fermentation) of **A)** acetate; **B)** butyrate; **C)** propionate; and **D)** total SCFA. *** $p < 0.001$, **** $p < 0.0001$.

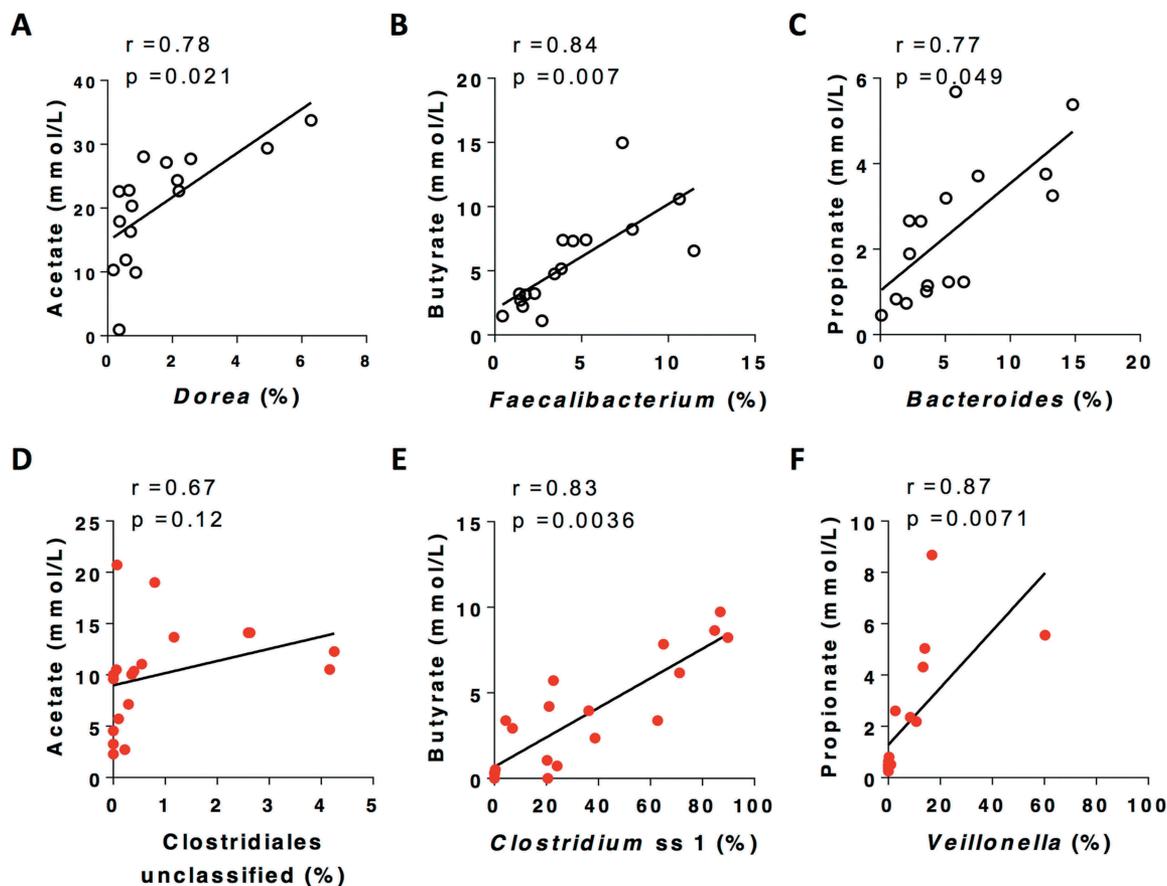


Figure 3. Representative taxa that were most strongly correlated with SCFA concentration post fermentation for non-CF control (black) and CF (red) samples (Full data of correlation between taxa and SCFA is included in Supplementary Figure 3). P values were from spearman correlation adjusted by Benjamini-Hochberg FDR procedure.

Clostridium sensu stricto cluster 1 (*Clostridium ss1*, $r = 0.83$, $p = 0.0036$, Figure 3E) and an unclassified member of the Clostridiales order ($r = 0.67$, $p = 0.0036$, Supplementary Figure 3). Propionate concentrations in CF reactions were significantly correlated with the relative abundance of the *Veillonella* genus ($r = 0.87$, $p = 0.0071$ Figure 3F). Acetate concentrations did not correlate significantly with any bacterial taxa in CF

reactions, although a positive trend with the unclassified member of the Clostridiales order was observed ($r = 0.67$, $p = 0.12$, Figure 3D). Taxa that showed positive correlations with SCFA production in healthy controls, including *Dorea*, *Anaerostipes*, *Faecalibacterium*, *Bacteroides*, were all significantly lower in post-fermentation CF samples (Supplementary Figure 4).

Inter-subject variation in SCFA production

HAMS fermentation reactions were highly reproducible, both in terms of SCFA production ($R = -0.0006$, $p = 0.42$, ANOSIM for experiment run 1 and run 2) and shifts in microbiota composition ($R = -0.037$, $p = 0.69$, ANOSIM for experiment run 1 and run 2). However, substantial inter-subject differences in SCFA biosynthesis were observed within both CF and control groups (Supplementary Figure 5A-H). CF and healthy control subjects were each divided into “high responder” (high-R) and “low responder” (low-R) groups according to changes in median acetate, butyrate, propionate and total SCFA concentrations during fermentation (Supplementary Figure 5A-H). In reactions involving faecal slurries

from healthy individuals, high butyrate production was associated with a high relative abundance of *Faecalibacterium* (Figure 4A). In contrast, low butyrate production was associated with a high relative abundance of *Clostridium* ss1 (Figure 4B), with a significant inverse correlation between these two taxa (Figure 4C). While a similar dichotomy was seen for CF samples, the bacterial taxa associated with high and low responder status were different. CF high-R samples (for both acetate and butyrate) were associated with a high relative abundance of *Clostridium* ss1, while CF low-R samples showed a high relative abundance of *Enterococcus* (Figure 4D-E). Again, a significant negative correlation was observed between these taxa ($r = -0.62$, $p = 0.005$, Figure 4F).

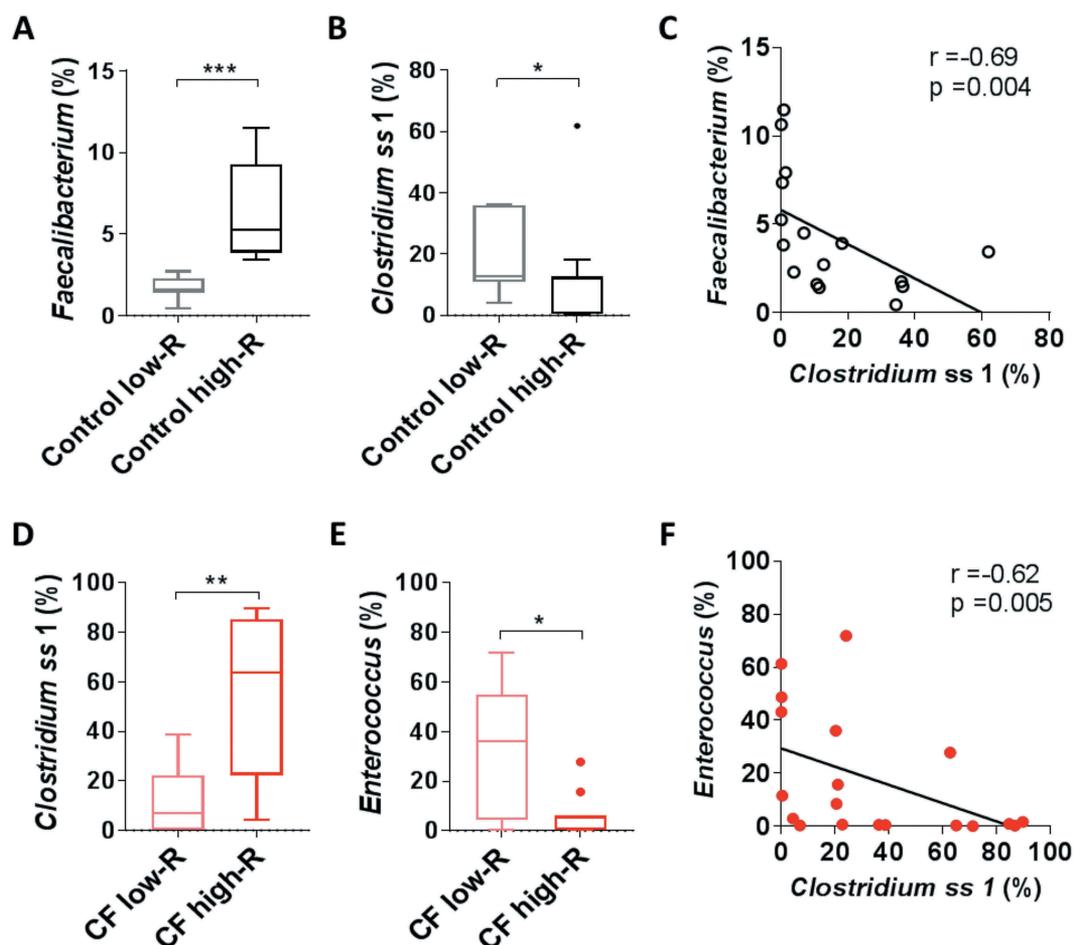


Figure 4. Butyrate production associated bacteria in low- and high- responder (low-R and high-R) control and CF reactions **A**) Relative abundance of *Faecalibacterium* in control low-R and high-R. **B**) Relative abundance of *Clostridium* ss1 in non-CF low-R and high-R. **C**) Correlation between relative abundance of *Clostridium* ss1 and *Faecalibacterium* (spearman) in non-CF sample (black). **D**) Relative abundance of *Clostridium* ss1 in CF low-R and high-R. **E**) Relative abundance of *Enterococcus* in CF low-R and high-R. **F**) Correlation between relative abundance of *Clostridium* ss1 and *Enterococcus* (spearman) in CF sample (red).

1H-NMR metabolomic analysis

Significant changes in metabolome composition occurred during fermentation in both CF slurry and healthy control slurry reactions (Supplementary Figure 6A-B). While metabolic pathways associated with HAMS fermentation were broadly comparable between CF and control groups (Supplementary Figure 6C-D), significant differences in relative abundance of specific metabolites were observed. Levels of acetate, formate, trimethylamine, and tyramine were all significantly higher in control post-fermentation metabolomes compared to CF reactions (Figure 5A-D). In contrast, the relative contributions of cystine, glycine, methionine, and proline to the metabolome were all significantly higher in post-fermentation CF reactions compared to control reactions (Figure 5E-H). Metabolites that were significantly elevated in CF reactions were all of relatively low abundance, with their elevation potentially reflecting lower levels of the high abundance metabolites, such as acetate and formate. Post-fermentation levels of lactate, a precursor of acetate, butyrate and propionate, trended higher for CF low-R compared to CF high-R, but did not achieve significance (median peak intensity, CF low-R: 260.9 [IQR: 110.2]; CF high-R: 26.5 [IQR:35.9], $p = 0.058$, Supplementary Fig. 7D).

Impact of healthy microbiota on SCFA biosynthesis in CF samples

The extent to which the addition of microbiota from healthy control samples could influence taxon predominance and SCFA production by CF faecal slurries was then investigated. The addition of microbiota from high-R control samples to high-R CF samples prior to fermentation did not, however, lead to the displacement of *Clostridium ss1* by *Faecalibacterium* as the taxon most closely correlated with post-fermentation butyrate levels (Supplementary Fig. 8). SCFA levels were also not changed significantly. The impact of introducing microbiota from high-R healthy control samples to low-R CF samples was then assessed. Again, despite the addition of material from samples that contained abundant fermentative commensal taxa, no significant increase in SCFA biosynthesis, or decrease in the post-fermentation relative abundance of *Enterococcus*, was observed (Figure 6A-B).

Discussion

We investigated the relative ability of faecal microbiota from adult CF patients and healthy non-CF controls to produce SCFAs through HAMS fermentation, and the bacterial taxa responsible. We report that the CF gut microbiota retains the

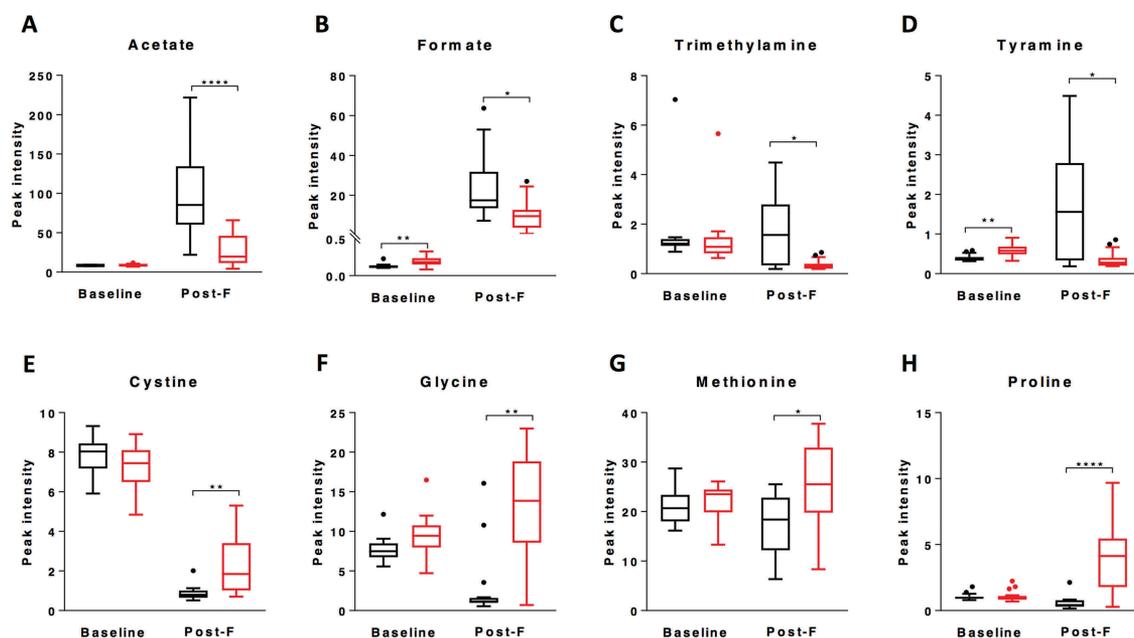


Figure 5. Metabolites that differed significantly between non-CF control (black) and CF (red) samples post HAMS fermentation (A-H). P values were from Mann-Whitney test adjusted by Benjamini-Hochberg FDR procedure. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

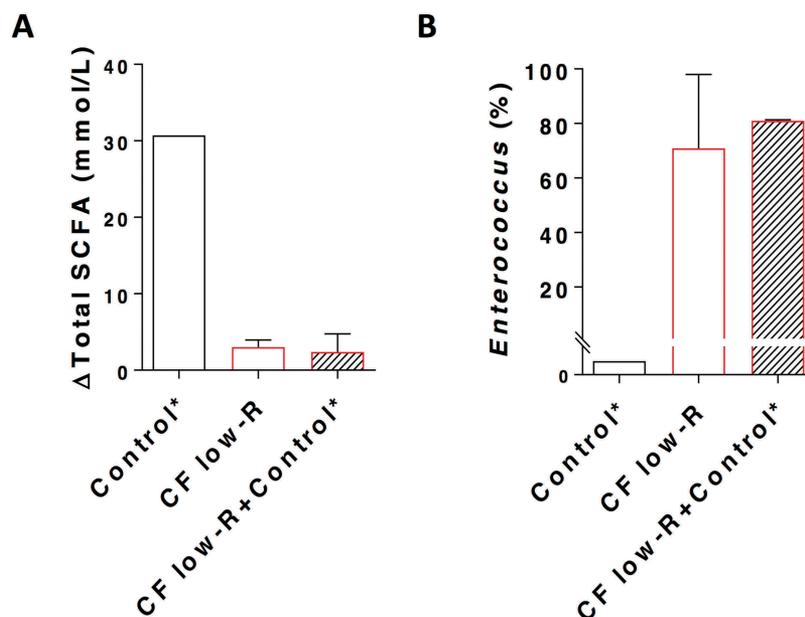


Figure 6. Total SCFA production (A) and relative abundance of *Enterococcus* (B) for CF low-Responder (low-R) faecal sample supplemented with non-CF control high-Responder (high-R) sample in a ratio of 1:0.1 of bacterial cells in the *in vitro* fermentation with HAMS. *Control is the combination of the top 3 non-CF control high-R.

ability to ferment HAMS in many cases, but that taxa that are characteristic of the altered CF faecal microbiota replace common commensal taxa in undertaking this role. In a subset of CF patients, the presence of HAMS leads to enterococcal overgrowth and the accumulation of lactate, suggesting that a prebiotic strategy would not be appropriate in all instances.

In keeping with previous analyses of the faecal microbiome in paediatric and adult CF patients^{11,14–17}, we found the relative abundance of common fermentative commensal taxa to be substantially reduced in CF subjects, compared to healthy adults. Particularly notable was the absence of important commensal taxa, such as *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *Ruminococcus bromii*. These are considered to be keystone species in the degradation of complex carbohydrates and the biosynthesis of SCFA in healthy individuals.^{37,38} Although diet is an important factor for shaping the gut microbiota, other factors, such as antibiotic exposure and disease related gastrointestinal dysfunction, contribute to an altered gut microbiota in patients with CF. To reflect the requirement for any potential clinical prebiotic approach to provide benefit despite variations in disease, diet, and antibiotic exposure, we conducted our analysis using samples collected

within the context of routine care. HAMS was selected as a fermentation substrate based on its potential as a candidate for clinical translation (in addition to its low cost, HAMS has Food and Drug Administration “generally regarded as safe” status).

Despite the low relative abundance of common fermentative taxa, SCFA biosynthesis was still evident in reactions using CF faecal slurries. Perhaps surprisingly, levels of butyrate and propionate production were not significantly lower than those obtained using healthy control slurries. This production of butyrate by the CF faecal microbiota was strongly associated with increases in the relative abundance of *Clostridium ss1*, a large cluster of *Clostridium* species that includes both commensal and pathogenic species.³⁹ Members of *Clostridium ss1* vary in their response to fermentation substrates, but exhibit a consistent capacity to synthesise butyrate.^{40,41} Clostridial abundance in CF faeces has been the focus of previous investigations, due to high rates of asymptomatic carriage of hypervirulent toxigenic strains of *Clostridium difficile* (*Clostridium* cluster XIa).⁴² The increased abundance of members of the *Clostridium* genus observed here was not unexpected and might reflect their ability to form spores that are highly resilient to

antibiotics. Indeed, reports of increasing *C. difficile* carriage rates^{43–47} possibly reflect increases in cumulative antibiotic exposure that accompany improved CF patient longevity.⁴⁸ In contrast, commensal members of the Clostridiales order that are not capable of sporulation, such as the *Faecalibacterium* and *Ruminococcus* genera, showed reduced relative abundance compared to healthy controls. *Clostridium* ss1 exhibited a trend towards negative correlation with butyrate production in healthy controls, in whom butyrogenesis was primarily associated with *Faecalibacterium*. These findings suggest a competitive relationship between *Clostridium* ss1 and *Faecalibacterium* in the fermentation of HAMS. In the absence of *Faecalibacterium* in CF patients, *Clostridium* ss1, where present, is able to exploit HAMS as a fermentation source, resulting in high levels of butyrate production.

Propionate levels following *in vitro* fermentation were not significantly lower in CF patients compared to healthy controls. However, the bacterial taxa that were associated with this process were again different. Rather than members of the genus *Bacteroides*, which were strongly associated with propionate levels in control reactions, propionate biosynthesis in CF reactions were associated with the relative abundance of members of the genus *Veillonella*. While most *Veillonella* species are unable to ferment carbohydrates, they can produce propionate by fermenting succinate produced by other taxa.⁴⁹ The replacement of *Bacteroides* by *Veillonella* in the CF microbiota might be due, in part, to the acquisition of antibiotic resistance by members of the *Veillonella* genus.^{49–51}

In contrast to butyrate and propionate, post-fermentation levels of acetate were significantly reduced in CF reactions compared with healthy controls. Acetate can be synthesised by a number of enteric bacteria⁵² mainly via the CoA-transferase pathway, involving the combined activities of phosphotransacetylase (PTA) and acetate kinase (AckA).⁵³ Acetate production in healthy controls was positively correlated with the relative abundance of *Anaerostipes* and *Dorea* genera; taxa that were substantially less prevalent in post-fermentation CF microbiota. The combination of reduced levels of genes encoding acetate

biosynthesis, and a low prevalence of taxa that are able to metabolise acetate (e.g. *Faecalibacterium*, *Roseburia* and *Coproccoccus*), suggests that acetate levels in CF reactions reflected reduced production, rather than increased bacterial utilisation.

To further understand the fermentation capacity of CF faecal microbiota, we compared the post-fermentation metabolome CF and healthy control reactions. The lower levels of acetate, and its intermediate formate, in CF reactions support our findings based on GC-FID results that CF has reduced capacity to produce acetate. Other metabolomic differences relating to non-starch components of the growth medium differed between CF and control reactions, including a reduced synthesis of trimethylamine and tyramine, and reduced utilisation of cystine and glycine, although these metabolites were observed in low abundance.

The failure of a subset of CF samples (CF low-R) to produce appreciable levels of SCFA was strongly associated with high *Enterococcus* relative abundance in post-fermentation microbiota. Enterococcal overgrowth is a common consequence of antibiotic exposure, in both animal models^{54,55} and humans.⁵⁶ Enterococci are capable of lactate biosynthesis and their high relative abundance was strongly associated with high lactate concentrations. Lactate can be further converted to acetate, butyrate and propionate by lactate-utilising bacteria.^{57,58} While this process does not typically occur at low pH (< 5.2)⁵⁷, post-fermentation pH did not differ significantly between CF low-R and high-R samples (data not shown). These findings suggest that lactate accumulation, observed in CF low-R reactions, where *Enterococcus* was predominant, resulted from reduced abundance of lactate-utilising taxa.

The implications of potential lactate accumulation on host physiology remain unclear. Luminal lactate has been shown to influence dendritic differentiation,⁵⁹ stimulate enterocyte hyper-proliferation in starvation-refed mice,⁶⁰ and to modulate LPS-dependent monocyte activation *in vitro*.^{61,62} Clinically, high faecal lactate concentrations have been reported in ulcerative colitis⁶³ and in chronic fatigue syndrome, along with an increased abundance of *Enterococcus faecalis*.⁶⁴ Our metagenomic analysis indicated that high

enterococcal abundance was also associated with the presence of genes capable of raffinose metabolism. Raffinose is a trisaccharide composed of galactose, glucose, and fructose, which is part of the FODMAP group of oligosaccharides.⁶⁵ Raffinose, is particularly abundant in leguminous seeds,⁶⁶ and the ability to degrade raffinose as a source of glucose might therefore provide an additional benefit in *Enterococcus*-dominated dysbiotic CF microbiota.

While our analyses suggest that HAMS supplementation in patients with CF would result in increased microbial production of SCFAs or lactate, it is important to also consider potential deleterious changes in intestinal microbiology. Increases in the relative abundance of *Clostridium ss1* might include potential pathogens, such as *C. perfringens*, while the *Enterococcus* genus contains species that are capable of causing serious infections, such as *E. faecium*. Asymptomatic carriage of toxigenic *C. difficile* is common in patients with CF and was detected in two of the CF samples analysed in this study, although the relative abundance of this pathogen did not increase in during HAMS fermentation (assessed by qPCR, data not shown). However, the potential for HAMS to confer a selective advantage on potential pathogens suggests dietary supplementation in CF patients would require careful microbiological monitoring.

Where prebiotic strategies fall short of their desired clinical impact, the low abundance of taxa that are able to exploit them might be a contributory factor. Interest in synbiotic approaches (preparations that combine prebiotics with viable commensal bacteria) therefore continues to grow. However, we found that the introduction of microbiota from healthy control samples had little impact on the microbiota composition or fermentation productivity of CF slurries. How *Clostridium ss1* or *Enterococcus* are able to remain numerically predominant during starch fermentation, even in the presence of genera such as *Faecalibacterium* and *Roseburia*, remains unclear. However, the out-competition of other bacteria during a rapid growth phase, combined with an ability to produce inhibitory compounds, such as bacteriocins,⁶⁷ might be contributory factors.

Our study had a number of limitations. We assessed stool, a sample that, while convenient, only approximates the intestinal microbiota. Furthermore, *in vitro* fermentation represents only a broad approximation of conditions within the colon.⁶⁸ Despite these limitations, we demonstrate that the highly dysbiotic CF gut microbiota retains the ability to exploit HAMS as a growth substrate, and a basis for SCFA production, in many cases. This ability is conferred by bacteria whose prevalence results from CF-associated changes in in the faecal microbiota. Manipulation of intestinal microbiota composition and function using dietary prebiotic supplementation offers exciting opportunities to achieve clinical benefit in CF. However, enterococcal overgrowth and lactate accumulation in microbiota from a subset of CF patients in response to HAMS suggests that a precision approach to clinical deployment would be most appropriate.

Conclusions

Despite significant alterations in composition, the adult CF gut microbiota retains the capacity to generate health-associated metabolites through HAMS fermentation. These findings suggest that the clinical potential of HAMS supplementation in adult CF patients warrants investigation. Our study also highlight that prebiotic effects can be mediated by taxa that are characteristic of disrupted microbial systems, despite ongoing perturbation, a finding that is contrary to the common assumptions that the persistence of commensal fermentative clades is necessary for such an effect. Finally, this study shows the potential for prebiotic effects to differ significantly depending on an individual's starting gut microbiota and underlines importance of personalised approaches to their deployment.

Materials and methods

Study population

This study was approved by Mater Hospital Ethics Board (HREC/15/MHS/131). Adult CF patients were recruited from the Mater Adult Cystic Fibrosis Clinic, Brisbane. Healthy non-CF adult

controls were recruited from Mater Hospital staff and associates. Subject characteristics are presented in Table 1. Stool samples were frozen and transported to the South Australian Health and Medical Research Institute, Adelaide, for analysis.

In vitro fermentation

To simulate gastric and small intestinal starch digestion, HAMS (Hylon VII, Ingredion ANZ, Pty Ltd) was subjected to *in vitro* pre-digestion (detailed in Supplementary Methods). Pre-digested HAMS was air-dried and UV-sterilised. The *in vitro* fermentation procedure was developed based on previously described method⁶⁹ with modifications. In brief, a basal medium (pH 6.8) containing tryptone (2.5g/L), yeast extract (0.5g/L), and mineral salts (detailed in Supplementary Methods) adapted from McSweeney *et al*⁷⁰ was used as fermentation medium. The medium was boiled and cooled under anaerobic conditions, prior to the addition of cysteine HCl. Fermentation medium (10 mL) was transferred to Hungate tubes (16 x 125 mm) under anaerobic conditions (10% CO₂ and 10% H₂ in N₂), which were stoppered and autoclaved. Faecal sample and pre-digested HAMS were dissolved in anaerobic diluent (pH 6.8)⁷⁰ to create a slurry. Faecal slurry (10% w/v) containing with 100 mg of faeces and

HAMS slurry (20% w/v) containing 100 mg of HAMS were injected to fermentation tubes under anaerobic conditions. Control fermentation tubes contained 500 µl of anaerobic diluent, in place of HAMS slurry. Hungate tubes were incubated at 37°C for 24 h with shaking. Fermentation medium and anaerobic diluent compositions are detailed in Supplementary Methods.

DNA extraction and 16S rRNA gene amplicon sequencing

DNA extractions were performed using a DNeasy PowerSoil HTP 96 DNA Isolation kit (Qiagen), as described previously.⁷¹ DNA concentration was quantified fluorometrically with a Quant-iT dsDNA Assay kit (Life Technologies). Amplicon sequencing of the V4 hypervariable region of the bacterial 16S rRNA gene was performed as described previously⁷¹ using an Illumina MiSeq platform. Paired-end 16S rRNA gene sequence reads were analysed with the Quantitative Insights into Microbial Ecology (QIIME) software (v1.8.0)⁷² following a previously published study.⁷¹ Operational taxonomic units (OTUs) were assigned to the reads using an open reference approach with UCLUST algorithm against the SILVA reference dataset (release 123)⁷³ that clustered at 97% identity. 16S rRNA gene sequence data and Metagenomic sequence data were submitted to the Sequence Read Archive under accession number SRP136140 and SRP136140, respectively. Negative controls were included in all DNA extraction, PCR amplification, and DNA sequencing processes.

Metagenomic sequencing

Metagenomic libraries were fragmented and indexed using Nextera XT DNA Library Prep Kit (Illumina Inc.), and Nextera XT Index kit (Illumina Inc.), respectively as per manufacturer's instructions. Amplicon libraries were then sequenced on the Illumina HiSeq 2500 platform at the SAHMRI David R Gunn Genomics Suite using Illumina HiSeq SBS 2 x 125bp v4 kit (Illumina Inc.). The sequencing resulted in an average of 18,586,781 ± 4,793,413 reads per sample. Bioinformatic processing of shotgun

Table 1. Study population characteristics.

	CF (n = 19)	Control (n = 16 ⁴)	P value
Age (years)	30 (19, 53)	37 (30, 55)	0.010 ²
Male (percentage)	47%	56%	> 0.99 ³
BMI	22.0 (18.1, 34.6)	26.2 (20.9, 31.3)	0.027 ²
ΔF508 homozygous (percentage)	53%	-	
ΔF508 heterozygous (percentage)	47%	-	
FEV1%	54 (28, 114)	-	
Pancreatic insufficiency (percentage)	95%	-	
Antibiotics for acute exacerbation within one month of sample collection	58%	0%	
Long-term maintenance antibiotics	95%	0%	

¹Values are in median (min, max) or percentage. ² P values were calculated from Mann-Whitney test. ³ P values were calculated from Fisher's exact test. ⁴ non-CF control group has 2 missing values for age, gender and BMI.

metagenomic sequence data was performed as described previously⁷⁴, with minor modifications (Supplementary Methods). Relative gene abundances were estimated by dividing the number of the gene-length normalized read counts for each gene by the total of reads from that sample that uniquely mapped to any gene in the catalogue. For analysis of functional capacity, genes annotated against the KEGG database were collapsed row-wise, based on their KEGG orthology identifiers, and summed for each pathway.

SCFA measurement

Pre- and post-fermentation samples were centrifuged at 13,000 g, 4 °C, for 10 min and supernatants collected for SCFA analysis. SCFA concentrations were measured by gas chromatography fitted with a flame ionisation detector (GC-FID, Flinders Analytical, Adelaide) using 4-Methylvaleric acid (Sigma-Aldrich) as internal standards.

Metabolomic analysis

Supernatants obtained from the *in vitro* fermentation were used for metabolomic analysis. Prior to the analysis, supernatants were frozen at -80°C , lyophilized at -58°C , and resuspended in 600 μl D_2O . Subsequently, ^1H NMR spectra were acquired under automation at 298 K and 700 MHz on a Bruker Avance II 700 NMR spectrometer (Bruker BioSpin) equipped with a 5-mm helium-cooled quadruple resonance cryoprobe and a cooled SampleJet sample changer.

Statistical analysis

Differences in alpha-diversity, SCFA, specific taxa, genes and metabolites between Control and CF groups were assessed using Mann-Whitney test (GraphPad Prism version 7.00). Group differences in microbiota beta diversity were assessed using the permutational analysis of variance (PERMANOVA) model with 9999 permutations based on the parameters permutation of residuals under a reduced model and a type III sum of squares (Primer-E v.7; Primer-E Ltd.).⁷⁵ Differences in taxa at the species level, generated from shotgun sequencing between groups at baseline, were analysed by linear

discriminant analysis (LDA) effect size (LEfSe) method.⁷⁶ Correlation between SCFA concentrations and taxa were performed using Spearman non-parametric correlation (GraphPad Prism). Benjamini-Hochberg false discovery rate control (BH-FDR), with a threshold of 0.05, was applied to analysis involving multi-testing. Differences in the metagenomics gene between groups were determined using Limma package in R⁷⁷, followed by pathway enrichment using MinPath.⁷⁸ Several criteria were applied; 1) a gene has to be detected in at least 60% of the samples, 2) a gene has to be significantly different between CF and healthy ($P \leq 0.05$, with $-2 \leq \text{Log}[\text{Fold Change}] \leq 2$), and 3) MiniPath-enriched pathways consisted of more than two genes. For metabolomic data, shifts of metabolites during the fermentation were presented in volcano plots with the indication of significantly changed metabolites determined by Mann-Whitney test adjusted with BH-FDR. Impacted pathways were analysed using pathway analysis by MetaboAnalyst 3.0⁷⁹

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Chmiel JF, Davis PB. State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? *Respir Res.* 2003;4:8. doi:10.1186/1465-9921-4-8.
- Steel A, Westaby D. Gastrointestinal disease in cystic fibrosis. In: Bush A, Bilton D, Hodson M, editors. *Hodson and Geddes' cystic fibrosis*. London: CRC Press; 2016. p. 302–317.
- Ooi CY, Lemberg DA, Day AS. Other causes of gastritis. *Pediatric Gastrointestinal Disease: B.C. Decker Inc.* 2008:165–174.
- Freeman AJ, Ooi CY. Pancreatitis and pancreatic cystosis in Cystic Fibrosis. *J Cyst Fibros.* 2017;16(Suppl 2):S79–S86. doi:10.1016/j.jcf.2017.07.004.
- Smyth RL, Croft NM, O'Hea U, Marshall TG, Ferguson A. Intestinal inflammation in cystic fibrosis. *Arch Dis Child.* 2000;82:394–399.
- Choi Y, Kim JW, Nam KH, Han SH, Kim JW, Ahn SH, Park DJ, Lee K-W, Lee HS, Kim -H-H. Systemic inflammation is associated with the density of immune cells in the tumor microenvironment of gastric cancer. *Gastric Cancer.* 2017;20:602–611. doi:10.1007/s10120-016-0642-0.
- Neglia JP, FitzSimmons SC, Maisonneuve P, Schoni MH, Schoni-Affolter F, Corey M, Lowenfels AB. The risk of cancer among patients with cystic fibrosis. *Cystic Fibrosis and Cancer Study Group. N Engl J Med.* 1995;332:494–499.
- Maisonneuve P, Marshall BC, Lowenfels AB. Risk of pancreatic cancer in patients with cystic fibrosis. *Gut.* 2007;56:1327–1328. doi:10.1136/gut.2007.125278.
- Schoni MH, Maisonneuve P, Schoni-Affolter F, Lowenfels AB. Cancer risk in patients with cystic fibrosis: the European data. *CF/CSG Group. J R Soc Med.* 1996;89(Suppl 27):38–43.
- De Rose V, Oliva A, Messori B, Grosso B, Mollar C, Pozzi E. Circulating adhesion molecules in cystic fibrosis. *Am J Respir Crit Care Med.* 1998;157:1234–1239. doi:10.1164/ajrccm.157.4.9704134.
- Duytschaever G, Huys G, Bekaert M, Boulanger L, De Boeck K, Vandamme P. Dysbiosis of bifidobacteria and *Clostridium* cluster XIVa in the cystic fibrosis fecal microbiota. *J Cyst Fibros.* 2013;12:206–215. doi:10.1016/j.jcf.2012.10.003.
- Scanlan PD, Buckling A, Kong W, Wild Y, Lynch SV, Harrison F. Gut dysbiosis in cystic fibrosis. *J Cyst Fibros.* 2012;11:454–455. doi:10.1016/j.jcf.2012.03.007.
- Hoffman LR, Pope CE, Hayden HS, Heltshe S, Levy R, McNamara S, Jacobs MA, Rohmer L, Radey M, Ramsey BW, et al. *Escherichia coli* dysbiosis correlates with gastrointestinal dysfunction in children with cystic fibrosis. *Clin Infect Dis.* 2014;58:396–399. doi:10.1093/cid/cit715.
- Manor O, Levy R, Pope CE, Hayden HS, Brittnacher MJ, Carr R, Radey MC, Hager KR, Heltshe SL, Ramsey BW, et al. Metagenomic evidence for taxonomic dysbiosis and functional imbalance in the gastrointestinal tracts of children with cystic fibrosis. *Sci Rep.* 2016;6:22493. doi:10.1038/srep22493.
- Duytschaever G, Huys G, Bekaert M, Boulanger L, De Boeck K, Vandamme P. Cross-sectional and longitudinal comparisons of the predominant fecal microbiota compositions of a group of pediatric patients with cystic fibrosis and their healthy siblings. *Appl Environ Microbiol.* 2011;77:8015–8024. doi:10.1128/AEM.05933-11.
- Schippa S, Iebba V, Santangelo F, Gagliardi A, De Biase RV, Stamato A, Bertasi S, Lucarelli M, Conte MP, Quattrucci S, et al. Cystic fibrosis transmembrane conductance regulator (CFTR) allelic variants relate to shifts in faecal microbiota of cystic fibrosis patients. *PLoS One.* 2013;8:e61176. doi:10.1371/journal.pone.0061176.
- Burke DG, Fouhy F, Harrison MJ, Rea MC, Cotter PD, O'Sullivan O, Stanton C, Hill C, Shanahan F, Plant BJ, et al. The altered gut microbiota in adults with cystic fibrosis. *BMC Microbiol.* 2017;17:58. doi:10.1186/s12866-017-0968-8.
- Nielsen S, Needham B, Leach ST, Day AS, Jaffe A, Thomas T, Ooi CY. Disrupted progression of the intestinal microbiota with age in children with cystic fibrosis. *Sci Rep.* 2016;6:24857. doi:10.1038/srep24857.
- Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell.* 2004;118:229–241. doi:10.1016/j.cell.2004.07.002.
- Murphy EA, Velazquez KT, Herbert KM. Influence of high-fat diet on gut microbiota: a driving force for chronic disease risk. *Curr Opin Clin Nutr Metab Care.* 2015;18:515–520. doi:10.1097/MCO.000000000000209.
- Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K, et al. A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut.* 2014;63:1275–1283. doi:10.1136/gutjnl-2013-304833.
- Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis.* 2015;26:26191.
- Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeiffer K, Coffey PJ, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature.* 2013;504:451–455. doi:10.1038/nature12726.
- Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci U S A.* 2014;111:2247–2252. doi:10.1073/pnas.1322269111.
- Saemann MD, Bohmig GA, Osterreicher CH, Burtscher H, Parolini O, Diakos C, Stöckl J, Hörl

- WH, Zlabinger GJ. Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB J.* 2000;14:2380–2382. doi:10.1096/fj.00-0359fje.
26. Freeland KR, Wolever TMS. Acute effects of intravenous and rectal acetate on glucagon-like peptide-1, peptide YY, ghrelin, adiponectin and tumour necrosis factor-alpha. *Br J Nutr.* 2010;103:460–466. doi:10.1017/S0007114509991863.
27. Higgins JA. Resistant starch: metabolic effects and potential health benefits. *J AOAC Int.* 2004;87:761–768.
28. Alvaro A, Sola R, Rosales R, Ribalta J, Anguera A, Masana L, Vallvé JC. Gene expression analysis of a human enterocyte cell line reveals downregulation of cholesterol biosynthesis in response to short-chain fatty acids. *IUBMB Life.* 2008;60:757–764. doi:10.1002/iub.110.
29. Wang HB, Wang PY, Wang X, Wan YL, Liu YC. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. *Dig Dis Sci.* 2012;57:3126–3135. doi:10.1007/s10620-012-2259-4.
30. Ruppin H, Bar-Meir S, Soergel KH, Wood CM, Schmitt MG Jr. Absorption of short-chain fatty acids by the colon. *Gastroenterology.* 1980;78:1500–1507.
31. Birt DF, Boylston T, Hendrich S, Jane JL, Hollis J, Li L, McClelland J, Moore S, Phillips GJ, Rowling M, et al. Resistant starch: promise for improving human health. *Adv Nutr.* 2013;4:587–601. doi:10.3945/an.113.004325.
32. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, Brown D, Stares MD, Scott P, Bergerat A, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *Isme J.* 2011;5:220–230. doi:10.1038/ismej.2010.118.
33. Phillips J, Muir JG, Birkett A, Lu ZX, Jones GP, O’Dea K, Young GP. Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *Am J Clin Nutr.* 1995;62:121–130. doi:10.1093/ajcn/62.1.121.
34. Le Leu RK, Winter JM, Christophersen CT, Young GP, Humphreys KJ, Hu Y, Gratz SW, Miller RB, Topping DL, Bird AR, et al. Butyrylated starch intake can prevent red meat-induced O6-methyl-2-deoxyguanosine adducts in human rectal tissue: a randomised clinical trial. *Br J Nutr.* 2015;114:220–230. doi:10.1017/S0007114515001750.
35. Aliasgharzadeh A, Dehghan P, Gargari BP, Asghari-Jafarabadi M. Resistant dextrin, as a prebiotic, improves insulin resistance and inflammation in women with type 2 diabetes: a randomised controlled clinical trial. *Br J Nutr.* 2015;113:321–330. doi:10.1017/S0007114514003675.
36. McLoughlin RF, Berthon BS, Jensen ME, Baines KJ, Wood LG. Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: a systematic review and meta-analysis. *Am J Clin Nutr.* 2017;106:930–945. doi:10.3945/ajcn.117.156265.
37. Louis P, Young P, Holtrop G, Flint HJ. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA: acetateCoA-transferase gene. *Environ Microbiol.* 2010;12:304–314. doi:10.1111/j.1462-2920.2009.02066.x.
38. Ze X, Duncan SH, Louis P, Flint HJ. *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. *Isme J.* 2012;6:1535–1543. doi:10.1038/ismej.2012.4.
39. Gupta RS, Gao B. Phylogenomic analyses of clostridia and identification of novel protein signatures that are specific to the genus *Clostridium sensu stricto* (cluster I). *Int J Syst Evol Microbiol.* 2009;59:285–294. doi:10.1099/ijs.0.001792-0.
40. Rainey FA, Hollen BJ, Small A. Genus I. *Clostridium* Prazmowski 1880, 23AL. In: Vos P, Garrity G, Jones D, editors. *Bergey’s manual of systematic bacteriology volume 3: the Firmicutes.* New York: Springer; 2009. p. 738–828.
41. Chen CK, Blaschek HP. Effect of acetate on molecular and physiological aspects of *Clostridium beijerinckii* NCIMB 8052 solvent production and strain degeneration. *Appl Environ Microbiol.* 1999;65:499–505.
42. Burke DG, Harrison MJ, Fleming C, McCarthy M, Shortt C, Sulaiman I, Murphy DM, Eustace JA, Shanahan F, Hill C, et al. *Clostridium difficile* carriage in adult cystic fibrosis (CF); implications for patients with CF and the potential for transmission of nosocomial infection. *J Cyst Fibros.* 2017;16:291–298. doi:10.1016/j.jcf.2016.09.008.
43. Wu TC, Fung JC. Evaluation of the usefulness of counterimmunoelectrophoresis for diagnosis of *Clostridium difficile*-associated colitis in clinical specimens. *J Clin Microbiol.* 1983;17:610–613.
44. Welton CJ, Long SS, Thompson CM Jr., Gilligan PH. *Clostridium difficile* in patients with cystic fibrosis. *Am J Dis Child.* 1985;139:805–808.
45. Peach SL, Borriello SP, Gaya H, Barclay FE, Welch AR. Asymptomatic carriage of *Clostridium difficile* in patients with cystic fibrosis. *J Clin Pathol.* 1986;39:1013–1018.
46. Yahav J, Samra Z, Blau H, Dinari G, Chodick G, Shmueli H. *Helicobacter pylori* and *Clostridium difficile* in cystic fibrosis patients. *Dig Dis Sci.* 2006;51:2274–2279. doi:10.1007/s10620-006-9271-5.
47. Pokorny CS, Bye PT, MacLeod C, Selby WS. Antibiotic-associated colitis and cystic fibrosis. *Dig Dis Sci.* 1992;37:1464–1468.
48. Hurley MN, McKeever TM, Prayle AP, Fogarty AW, Smyth AR. Rate of improvement of CF life expectancy exceeds that of general population—observational death registration study. *J Cyst Fibros.* 2014;13:410–415. doi:10.1016/j.jcf.2013.12.002.
49. Rainey FA. Family X. *Veillonellaceae* Rogosa 1971b, 232AL. In: Vos P, Garrity G, Jones D, editors. *Bergey’s manual of systematic bacteriology volume 3: the Firmicutes.* New York: Springer; 2009. p. 1059–1129.

50. Ready D, Pratten J, Roberts AP, Bedi R, Mullany P, Wilson M. Potential role of *Veillonella* spp. as a reservoir of transferable tetracycline resistance in the oral cavity. *Antimicrob Agents Chemother.* 2006;50:2866–2868. doi:10.1128/AAC.00217-06.
51. Worlitzsch D, Rintelen C, Bohm K, Wollschlager B, Merkel N, Borneff-Lipp M, Döring G. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. *Clin Microbiol Infect.* 2009;15:454–460. doi:10.1111/j.1469-0691.2008.02659.x.
52. Flint HJ, Duncan SH, Scott KP, Louis P. Links between diet, gut microbiota composition and gut metabolism. *Proc Nutr Soc.* 2015;74:13–22. doi:10.1017/S0029665114001463.
53. Ferry JG. Acetate kinase and phosphotransacetylase. *Methods Enzymol.* 2011;494:219–231. doi:10.1016/B978-0-12-385112-3.00011-1.
54. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van Den Brink MRM, Kamboj M, et al. Vancomycin-resistant *Enterococcus* domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. *J Clin Invest.* 2010;120:4332–4341. doi:10.1172/JCI43918.
55. Hendrickx AP, Top J, Bayjanov JR, Kemperman H, Rogers MR, Paganelli FL, Bonten MJM, Willems RJL. Antibiotic-Driven dysbiosis mediates intraluminal agglutination and alternative segregation of *enterococcus faecium* from the intestinal epithelium. *MBio.* 2015;6:e01346–15. doi:10.1128/mBio.01346-15.
56. Hufnagel M, Liese C, Loescher C, Kunze M, Proempeler H, Berner R, Krueger M. Enterococcal colonization of infants in a neonatal intensive care unit: associated predictors, risk factors and seasonal patterns. *BMC Infect Dis.* 2007;7:107. doi:10.1186/1471-2334-7-89.
57. Belenguer A, Duncan SH, Holtrop G, Anderson SE, Lobley GE, Flint HJ. Impact of pH on lactate formation and utilization by human fecal microbial communities. *Appl Environ Microbiol.* 2007;73:6526–6533. doi:10.1128/AEM.00508-07.
58. Bourriaud C, Robins RJ, Martin L, Kozlowski F, Tenailleau E, Cherbut C, Michel C. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. *J Appl Microbiol.* 2005;99:201–212. doi:10.1111/j.1365-2672.2005.02605.x.
59. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, Mackensen A, Kreutz M. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood.* 2006;107:2013–2021. doi:10.1182/blood-2005-05-1795.
60. Okada T, Fukuda S, Hase K, Nishiumi S, Izumi Y, Yoshida M, Hagiwara T, Kawashima R, Yamazaki M, Oshio T, et al. Microbiota-derived lactate accelerates colon epithelial cell turnover in starvation-refed mice. *Nat Commun.* 2013;4:1654. doi:10.1038/ncomms2668.
61. Dietl K, Renner K, Dettmer K, Timischl B, Eberhart K, Dorn C, Hellerbrand C, Kastenberger M, Kunz-Schughart LA, Oefner PJ, et al. Lactic acid and acidification inhibit TNF secretion and glycolysis of human monocytes. *J Immunol.* 2010;184:1200–1209. doi:10.4049/jimmunol.0902584.
62. Peter K, Rehli M, Singer K, Renner-Sattler K, Kreutz M. Lactic acid delays the inflammatory response of human monocytes. *Biochem Biophys Res Commun.* 2015;457:412–418. doi:10.1016/j.bbrc.2015.01.005.
63. Vernia P, Caprilli R, Latella G, Barbetti F, Magliocca FM, Cittadini M. Fecal lactate and ulcerative colitis. *Gastroenterology.* 1988;95:1564–1568.
64. Sheedy JR, Wettenhall RE, Scanlon D, Gooley PR, Lewis DP, McGregor N, Stapleton DI, Butt HL, DE Meirleir KL. Increased d-lactic Acid intestinal bacteria in patients with chronic fatigue syndrome. *In Vivo.* 2009;23:621–628.
65. Gibson PR, Shepherd SJ. Personal view: food for thought—western lifestyle and susceptibility to Crohn’s disease. The FODMAP Hypothesis. *Aliment Pharmacol Ther.* 2005;21:1399–1409. doi:10.1111/j.1365-2036.2005.02506.x.
66. Tester RF, Karkalas J. Carbohydrates: classification and properties. In: Trugo L, Finglas PM, editors. *Encyclopedia of food sciences and nutrition.* Cambridge (MA): Academic Press; 2003. p. 862–875.
67. Ness IF, Diep DB, Ike Y. Enterococcal bacteriocins and antimicrobial proteins that contribute to niche control. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. *Enterococci: from commensals to leading causes of drug resistant infection.* Boston: Massachusetts Eye and Ear Infirmary; 2014.
68. Long W, Xue Z, Zhang Q, Feng Z, Bridgewater L, Wang L, Zhao L, Pang X. Differential responses of gut microbiota to the same prebiotic formula in oligotrophic and eutrophic batch fermentation systems. *Sci Rep.* 2015;5:13469. doi:10.1038/srep13469.
69. Edwards CA, Gibson G, Champ M, Jensen BB, Mathers JC, Nagengast F, Rumney C, Quehl A. In vitro method for quantification of the fermentation of starch by human faecal bacteria. *J Sci Food Agr.* 1996;71:209–217. doi:10.1002/(ISSN)1097-0010.
70. McSweeney CS, Denman SE, Mackie RI. Rumen bacteria. In: Makkar HPS, McSweeney CS, editors. *Methods in gut microbial ecology for ruminants.* Dordrecht: International Atomic Energy Agency; 2005. p. 23–37.
71. Choo JM, Leong LE, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. *Sci Rep.* 2015;5:16350. doi:10.1038/srep16350.
72. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* 2010;7:335–336. doi:10.1038/nmeth.f.303.

73. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 2013;41: D590–D6. doi:10.1093/nar/gks1219.
74. Nielsen HB, Almeida M, Juncker AS, Rasmussen S, Li J, Sunagawa S, Plichta DR, Gautier L, Pedersen AG, Le Chatelier E, et al. Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nat Biotechnol.* 2014;32:822–828. doi:10.1038/nbt.2939.
75. Anderson MJ, Walsh DCI. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecol Monogr.* 2013;83:557–574. doi:10.1890/12-2010.1.
76. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. *Genome Biol.* 2011;12:R60. doi:10.1186/gb-2011-12-10-r102.
77. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43:e47. doi:10.1093/nar/gkv007.
78. Ye Y, Doak TG. A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Comput Biol.* 2009;5:e1000465. doi:10.1371/journal.pcbi.1000509.
79. Xia J, Wishart DS. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Curr Protoc Bioinformatics.* 2016;55:14 0 1–0 91. doi:10.1002/cpbi.11.