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Cystic Fibrosis Related Gut Dysbiosis: A Systematic Review

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Abstract

Background & Aims: Cystic Fibrosis (CF) is associated with gut dysbiosis, local and systemic inflammation and impaired immune function. Gut microbiota dysbiosis results from changes in the complex gut milieu in response to CF transmembrane conductance regulator (CFTR) dysfunction, pancreatic malabsorption, diet, medications and environmental influences. In several diseases, alteration of the gut microbiota influences local and systemic inflammation and disease outcomes. We conducted a systematic review of the gut microbiota in CF and explored factors influencing dysbiosis.

Methods: An electronic search of three databases was conducted in January 2019, and re-run in June 2021. Human, animal and *in vitro* studies were included. The primary outcome was differences in the gut microbiota between people with CF (pwCF) and healthy controls. Secondary outcomes included the relationship between the gut microbiota and other factors, including diet, medication, inflammation and pulmonary function in pwCF.

Results: Thirty-eight studies were identified. The literature confirmed the presence of CF-related gut dysbiosis, characterised by reduced diversity and several taxonomic changes. There was a relative increase of bacteria associated with a pro-inflammatory response coupled with a reduction of those considered anti-inflammatory. However, studies linking gut dysbiosis to systemic and lung inflammation were limited. Causes of gut dysbiosis were multifactorial, and findings were variable. Data on the impact of CFTR modulators on the gut microbiota was limited.

Conclusions: CF-related gut dysbiosis is evident in pwCF. Whether this influences local and systemic disease and is amenable to interventions with diet and drugs, such as CFTR modulators, requires further investigation.

Word count: 249

Key words:

Cystic fibrosis, gut microbiota, inflammation, diet, dysbiosis, CFTR

Introduction

The gut microbiota influences human health and disease and plays an important role in nutrition, metabolism, immunity, inflammation and malignancy. The term gut microbiota refers to the collective communities of microorganisms residing within the gut, including bacteria, fungi, viruses, protozoa, archaea, and parasites (see Table 1 for key definitions) [1]. In this review, we focus on the gut bacteriome, which has been relatively well characterised in industrialized societies and includes over 1000 species of bacteria residing in the human gut, which can vary widely between healthy individuals as well as in those with various disease states [2]. While some gut bacteria are pathobionts, others are commensal or even symbiotic, with different species from various phyla involved in varied symbiotic relationships with both the host and other bacteria. Many species however fall within a grey zone in which environmental and host factors as well as species abundance and overall gut microbiome composition determines whether they are considered beneficial, neutral or detrimental.

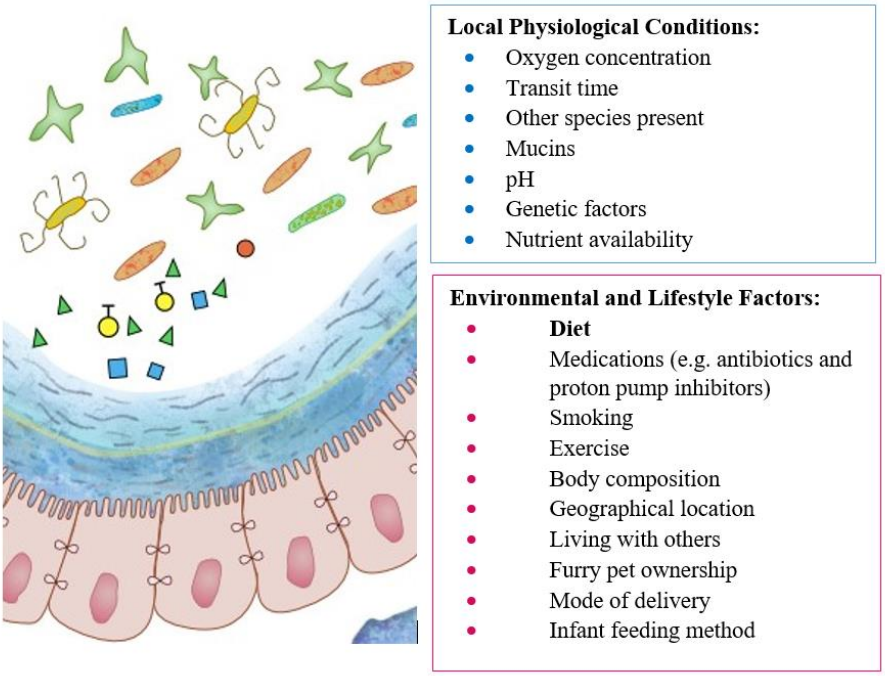
Table 1 Key Terminology

Term	Definition
Alpha-diversity	The microbial diversity within an individual sample. It is subdivided into species richness, evenness and diversity
Beta-diversity	A measure of how different the microbiota composition is between samples. For example, between different individuals or different samples from the same individual
Bray-Curtis dissimilarity	An abundance-based beta-diversity metric which does not incorporate phylogenetic information except in terms of the phylogenetic level chose to define groups (strain, species, genus, family, etc.)
Cystic Fibrosis Related Gut Dysbiosis	The imbalance in gut microbiota frequently associated with condition cystic fibrosis
Dysbiosis	An imbalance in the microbiota
Lipopolysaccharide	A component of the cell membrane of gram-negative bacteria
Metabolomics	Analysis of microorganisms' metabolic products
Microbiome	Total genetic material of a microbial community
Microbiota	Specific communities of microorganisms
Species diversity	A measure of alpha-diversity, the relative abundance of different species within a community or sample, which is mainly comprised of species evenness and richness
Species Evenness	An alpha-diversity metric of how evenly the species are distributed within that sample
Species Richness	An alpha-diversity metric, measuring the number of species present within a sample
Unifrac distance	A beta-diversity metric which incorporates phylogenetic information [3]. Weighted Unifrac distance also takes into account the taxa or species' relative abundance

Anatomically, there is notable variation in the density and composition of gut bacterial communities owing to regional differences in the gut physiological environment and transit times, with a significantly higher number of bacteria residing in the colon. The gut microbiota composition varies throughout life and is influenced by numerous factors including the mode of delivery, infant feeding, disease states, diet, medication, smoking and exercise (Fig. 1) [4].

The gut microbiota should therefore be considered as a distinct, metabolically active, symbiotic organ which regulates key host functions including metabolism, absorption, innate and adaptive immunity as well as local and systemic inflammation. Changes which result in a gut microbiota imbalance (dysbiosis) can have a profound effect on both intestinal and extra-intestinal disorders and may influence disease outcome in conditions such as cystic fibrosis (CF)**.**

Figure 1 Key factors influencing gut microbiota composition



Cystic fibrosis results from the abnormal production and function of the cystic fibrosis transmembrane conductance regulator (CFTR), a protein which is expressed throughout the body [5, 6]. Aberrations of the CF gut physiology may accentuate dysbiosis due to a complex milieu of altered intestinal mucus secretion, abnormal luminal pH and nutrient availability, pancreatic malabsorption, high fat, low fibre diet, frequent use of antibiotics and proton pump inhibitors (PPI) [7-14]. The microbiota perturbances and changes in microbe-derived

components and metabolites may further exacerbate the already exaggerated local and systemic pro-inflammatory state reported in people with CF (pwCF) [15-18].

Colonic bacterial imbalance has the potential of accentuating inflammation through the production of immuno-stimulating bacterial components, such as lipopolysaccharide (LPS) derived from gram-negative bacteria (such as *Escherichia coli and Veillonella parvula)*, glucorhamnan (a polysaccharide produced by *Faecalicatena gnavus*) and glucosylating and binary toxins [19, 20]. Lipopolysaccharides are known to activate toll-like receptor 4 (TLR4) and upregulate inflammatory pathways, including nuclear factor kappa B (NF-KB) and NLRP3 with the release of inflammatory cytokines, such as interleukin (IL)-6, tumour necrosis factor (TNF)-α, IL-18 and IL-1β [20, 21]. In contrast, *Bacteroides*' LPS can inhibit TLR4 either alone or prior to stimulation by *Escherichia coli* (*E. coli*) [22, 23]. Similarly, certain bifidobacterial strains can downregulate LPS-induced NF-KB activation and reduce IL-8 levels *in vitro* [24]. A shift in the ratio of immuno-stimulatory and inhibitory LPS may influence both local and systemic inflammation and thereby influence lung health, Fig 2.

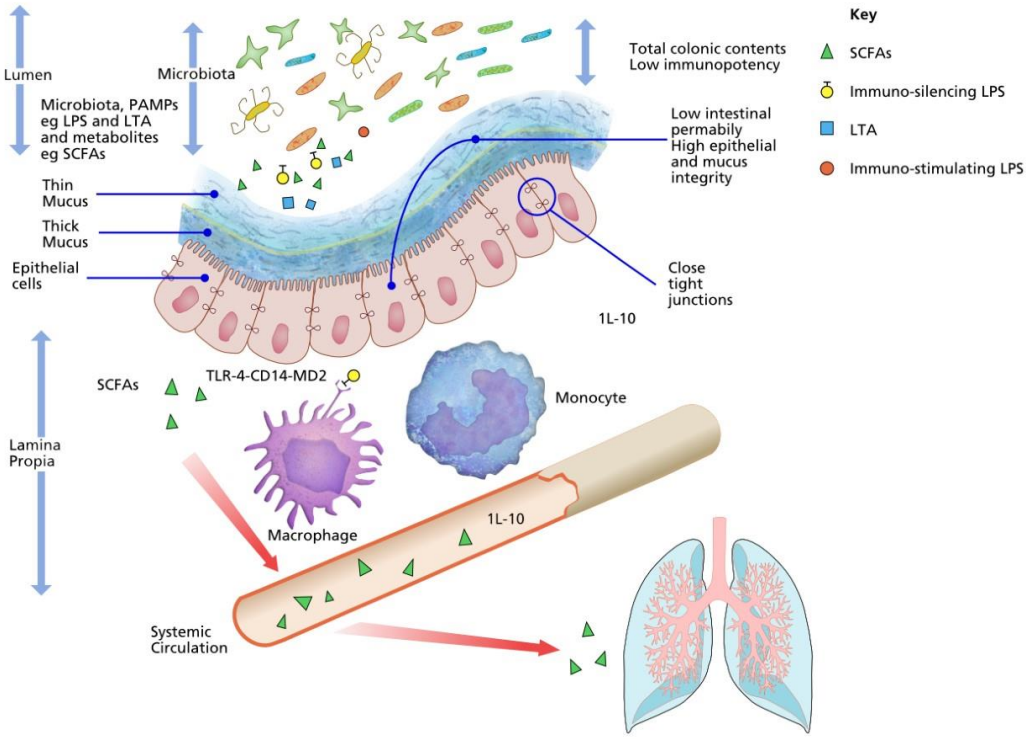
Certain bacteria can also exert important anti-inflammatory properties through the production of SCFAs, particularly butyrate, following fermentation of carbohydrates [25]. Butyrate upregulates MUC2 mRNA levels, the main gut mucin, thereby protecting epithelial cells from direct contact with intestinal bacteria [26, 27] and, in reverse, potentially protecting extremely oxygen sensitive commensal species from oxygen diffusion into the gut [28]. SCFAs augment the integrity of the intestinal barrier by increasing the assembly of epithelial tight junctions [29] and stabilisation of hypoxic induced factor (HIF) which coordinates barrier protection [28]. There has been growing interest in the immune modulatory role of SCFAs in diseases, such as asthma [30], where they reduce IL-6 and TNF-α release from macrophages and neutrophils, enhance macrophages' antimicrobial activity and increase the production and function of regulatory T cells (Tregs) [31, 32]. The immune effects of SCFAs are far-reaching

and in a mouse model, butyrate supplementation was found to protect against influenza by attenuating neutrophil infiltration and increasing CD8+ T cell metabolism [33]. The balance of different bacteria is particularly important in determining whether, the overall bacteriome promote a pro-or anti-inflammatory state (Fig. 2).

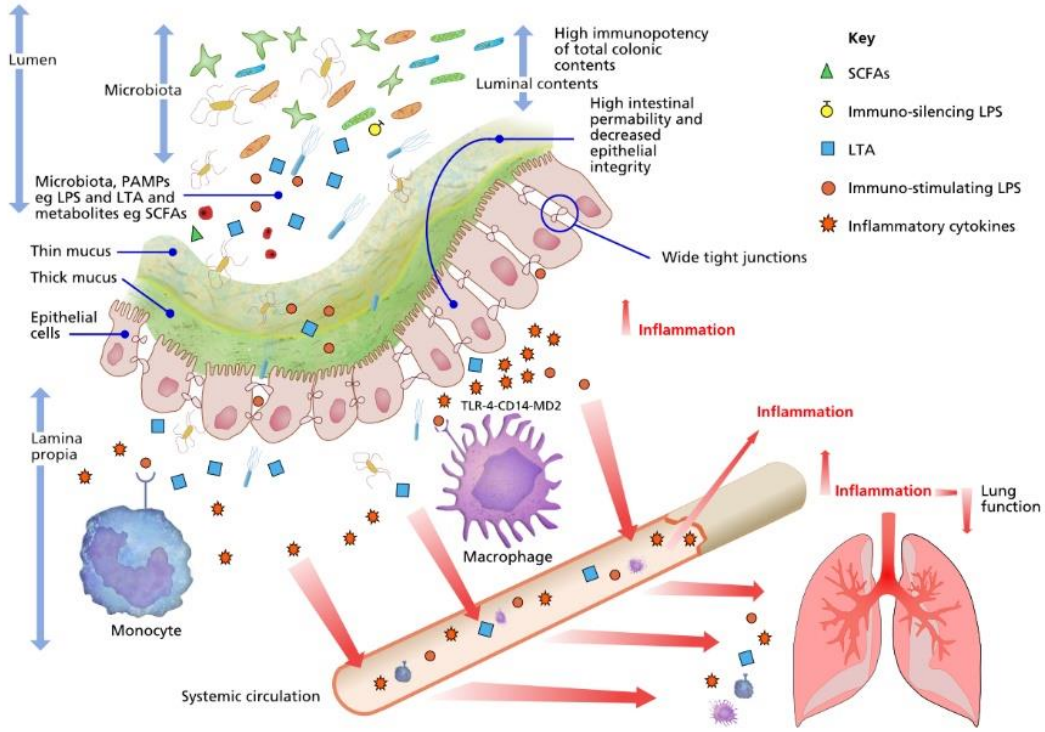
While there is increasing literature supporting the presence of CF related gut dysbiosis (CFRGD), the underlying aetiology and drivers of dysbiosis as well as the impact of CFRGD on inflammation, gastrointestinal (GI) symptoms and lung health remains unclear. Identifying factors, such as diet and drugs, which may positively influence the gut microbiota in pwCF, may help improve both local and systemic disease. In this systematic review, we aimed to determine which factors influence gut dysbiosis in CF.

Figure 2: Mechanisms whereby the gut microbiota modulate gut, systemic and lung inflammation (a) in health and (b) disease states

a)



b)



Methods

This systematic review was performed in accordance with the PRISMA (Preferred Reporting Items for Systematic Review and Meta-analyses) guidelines [34] and was prospectively registered with the PROSPERO database (CRD42019124513) [35].

Search Strategy

Web of Science, MEDLINE and EMBASE were searched from January 2019 through June 2021. The search terms were: 'cystic fibrosis' AND 'gut microbiota' OR 'gut microbes' OR 'gut microbiome' OR 'gut dysbiosis' OR 'dysbiotic gut' OR 'gut flora' OR 'gut microflora' OR 'intestinal microbiota' OR 'intestinal microbiome'. The reference list of all included papers were screened and the Cochrane library was searched January 2019.

Eligibility criteria

For inclusion, studies were required to meet the following criteria: participants of any age in humans with CF or CF-animal models or CF *in vitro* studies investigating the colonic gut microbiota. All study designs were considered. Interventional studies were only included when pre- or without intervention data could be isolated, to assess the gut microbiota under normal clinical conditions. Studies were included if they were published in peer-review journals. All studies were required to at least contain a measure of the colonic gut microbiota in pwCF or a CF animal or in an *in vitro* model. No date limits were set.

Studies were excluded if they (a) only measured the small intestinal intestinal bacteria - because this is distinct to the resident colonic microbiota (b) were not published in the English language, due to resource limitations. Review articles, meta-analyses, case studies, conference proceedings/abstracts, book chapters, and unpublished theses were not included. Finally, studies with critically unwell or mechanically ventilated participants fell outside the review's scope.

Screening

Search results were compiled in the referencing software programme Endnote™ X9 (Clarivate, Philadelphia) and duplicates were removed. The titles of all papers were screened and, if relevant, the abstract and then full paper was reviewed against the eligibility criteria. Two researchers (LC and DP) independently assessed studies for inclusion and later compared notes to reach a mutual consensus. Disagreements about the eligibility of any particular study was resolved by a third reviewer (HW). Potential studies that could be included based on their title or abstract were retrieved in full-text and reviewed against the inclusion/exclusion criteria independently by two researchers (LC and DP) with a third researcher (HW) used to settle any disputes.

Summary Outcome Measures

The primary outcome was the colonic gut microbiota in CF compared to healthy controls (HCs). Secondary outcomes included gut microbiota functionality, short chain fatty acid (SCFA) levels and the interaction between the gut microbiota and factors, including CF genotype, pancreatic status (sufficient or insufficient), CF-related liver disease (CFRLD), GI symptoms, diet, medication, inflammation, and pulmonary outcomes, in pwCF.

Data Extraction and Critical Appraisal

Data were extracted from included papers using a standardized data collection form based on the Cochrane template [36]. Each paper was critically appraised. For human studies, Law et al.'s 'Guidelines for Critical Review Form for Quantitative Studies' was selected [37]. To ensure both breath and specificity, important design-specific considerations from other tools were added to it, including questions from the STROBE checklist pertaining to methods of recruitment, consideration of confounders and appropriate statistical analysis and relevant questions regarding outcome measurements, intervention and results for randomised control

trials and cohort studies from the Joanna Briggs Institute critical appraisal tools and Cochrane assessment of bias [38-42]. For animal studies, the SYRCLE's Risk of Bias tool was selected [43] Critical appraisal summary scores were deemed inappropriate given the heterogeneity in study designs. Two reviewers undertook data extraction and critical appraisal (LC and HW).

Synthesis of results

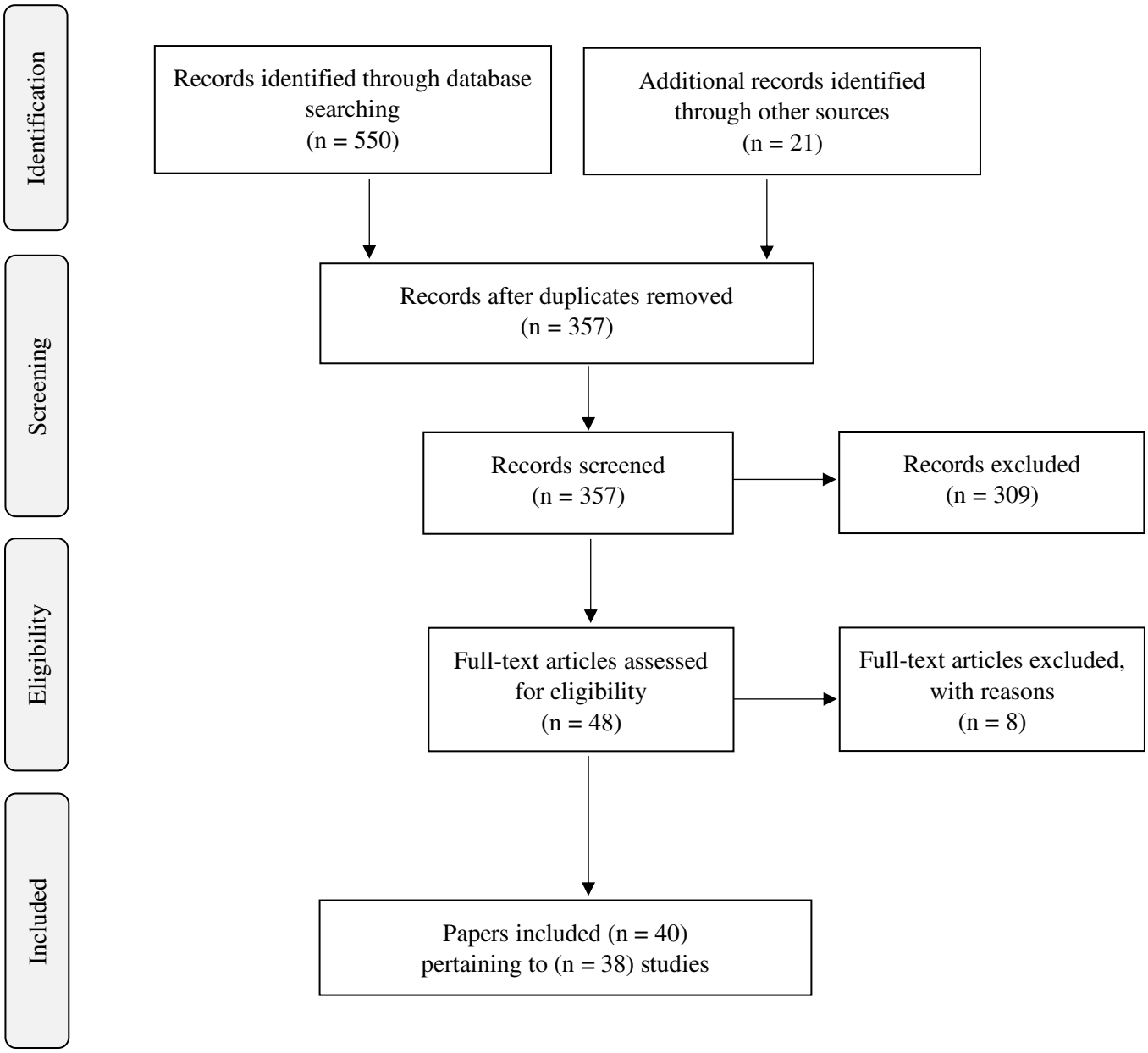
The results were synthesised by constructing a descriptive summary of the included studies in table form, including key concerns with bias, alongside a narrative synthesis [44]. Meta-analysis was not performed given the studies' heterogeneity.

Results

Search results

After removing duplicates, a total of 357 articles were identified on initial search with 48 full papers retrieved for detailed evaluation (Fig. 3). Of these, 38 studies were included in the final review (Fig. 3), two of which were on CF-mouse models [12, 45] and one CF-rabbit model [46] (Supplementary Table 1). One study utilised human adult faecal samples in an *in vitro* intervention study [47] and the remaining studies were in humans, predominately employing a cross-sectional or cohort design, (Supplementary Table 1). Of the 35 studies in humans, three were in infants (≤12 months) [48-50], six in infants and children [51-56], seven in children [57-63], five in children and adolescents [64-69], seven in children and adults [70-76] and seven studies were in adults with CF [47, 77-83]. Supplementary Table 1 summarises studies' key characteristics.

Figure 3: PRISMA 2009 Flow Diagram: CF and Gut Microbiota [84]



Gut Microbiota Analysis Methods

The most common gut microbiota analysis method was 16s rRNA, which was carried out in 31 studies, Supplementary Table 1, using a variety of different techniques (including pyrosequencing, Denaturing Gradient Gel Electrophoresis and MiSeq) [12, 45-48, 50, 51, 53-62, 65-73, 75-77, 79, 80, 83]. There was significant variation between studies in the sequenced hypervariable region of 16S rRNA selected (for example, [58, 72, 77, 83]). Other analytical methods included real-time polymerase chain reaction, faecal proteomics and whole genomic sequencing, Supplementary Table 1 [49, 52, 57, 64-67, 71, 78, 82]. Despite the variety of methods employed, clear microbiota differences in CF samples emerged.

Cystic Fibrosis Related Gut Dysbiosis

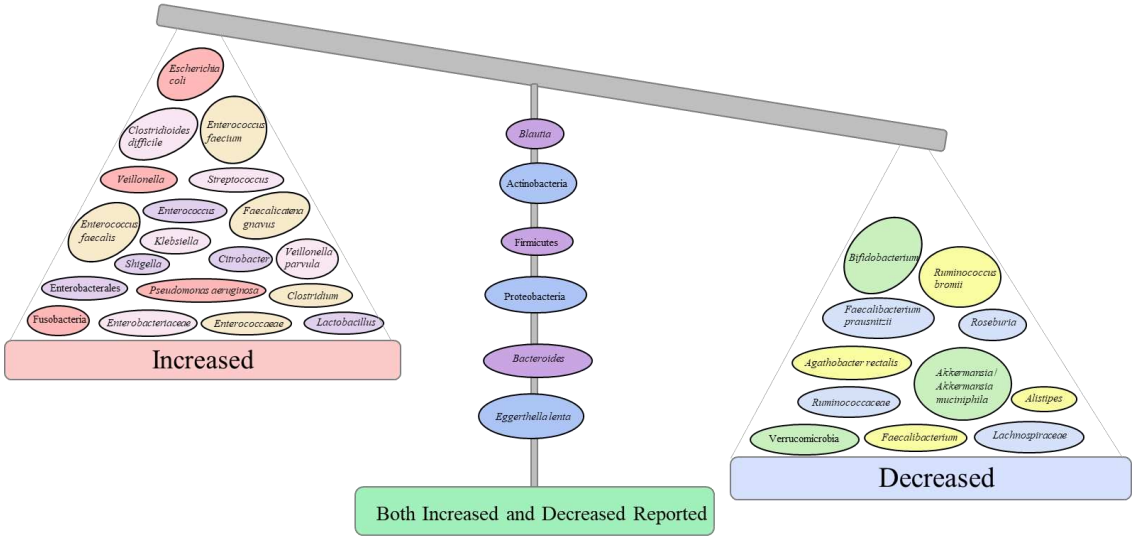
Studies confirm the presence of CF related gut dysbiosis (CFRGD) with the CF gut microbiota differing significantly from non-CF controls in terms of diversity, composition, and functionality.

Pre-weaning, infants with CF were often reported to have a higher faecal microbiota alpha-diversity when compared to healthy controls [48, 50]. A rapid increase in alpha diversity was reported post weaning in healthy controls while in CF infants the degree of change was more limited, with lower alpha-diversity being reported in infants, children and adults with CF [47-49, 51, 57, 58, 68, 71, 77, 83]. From a beta-diversity perspective, children and adults with CF showed a wider variety of (dysbiotic) compositions than healthy controls (the beta diversity within the CF group was larger than within healthy controls) [47, 58, 62, 68, 77, 83].

Certain key bacteria were frequently reported as having an altered relative abundance in pwCF as compared to HCs (Fig 4). An increase in the relative abundance of *Enterobacteriaceae, including E. coli* [49, 50, 58, 62, 64], Fusobacteria [58, 77] and species typically associated with the small intestine, such as *Lactobacillus* [62, 78], *Streptococcus* [48, 50, 62, 68],

Veillonella [51, 58, 68], *Enterococcus faecalis* [47, 51, 78] and *Enterococcus faecium* [47, 51] were reported in multiple studies (Fig. 4 and Supplementary Table 2). In contrast, the relative abundance of various commensal and potentially beneficial bacteria were typically decreased in pwCF. These included *Bifidobacterium* [50, 57, 83], *Akkermansia* [50, 53, 62] and butyrate-producers, such as *Faecalibacterium prausnitzii* [47, 48, 57, 71, 83] and *Roseburia* [62, 64, 83] (Fig. 4 and Supplementary Table 2). Some commensal genera, like *Bacteroides* and *Blautia*, were reported to be either increased or decreased, depending on the study ([48-50, 57, 62-64, 68, 71, 77, 78, 83]. These are typically species associated with the *Bacteroides* enterotype.

Figure 4 Summary of differences in bacterial relative abundance between people with cystic fibrosis and healthy controls from studies identified in the literature search



[47-51, 53, 57, 58, 61-66, 68, 71, 76-78, 83]

Gut Microbiota Functionality

Gut microorganisms modulate host metabolism and specific metabolic pathways as measured by enzyme activities or coupling the relative abundances of regulatory gene expression with metabolic pathways. Both an increase and decrease in carbohydrate and lipid metabolism pathway abundances were reported in CF (Supplementary Table 3) [58, 61, 78]. Several studies identified enriched amino acid degradation pathways with a decrease in vitamin biosynthesis and metabolism pathways (Supplementary Table 3) [58, 77, 78]. Catabolism of the SCFAs

butyrate was reported to be increased [51] while propionate metabolism pathway abundances were reported to be both increased and decreased in the CF gut microbiota (Supplementary Table 3) [58, 77]. Fatty acid biosynthesis functional pathway was also found to be depleted in samples from young children with CF compared to non-CF samples [51].

Xenobiotic substances can also influence the gut microbiota's composition and metabolic activity, with numerous xenobiotic degradation pathways, including the meta-cleavage pathway of aromatic compounds, appearing to be enriched in CF stool when compared to HCs (Supplementary Table 3) [78]. *In vitro* testing of *Enterobacteriaceae* bacteria from CF stool showed higher resistance to amoxicillin [67]. A single study investigating the faecal resistome, identified a high proportion of aminoglycoside resistance in samples from adults with CF compared to HCs with almost a third of Enterobacterales isolates being resistant to tobramycin and gentamycin in samples from pwCF, compared to none in HCs [82].

Short Chain Fatty Acid Levels

Two studies measured SCFA levels. The first study found that non-CF children (n=31) had higher levels of acetate, butyrate and propionate compared to a similar cohort of children with CF [62]. In contrast, an *in vitro* fermentation model of faecal slurry from 16 HC and 19 adults with CF, found that only acetate levels were reduced in CF [47].

Genotype and CF-related Gut Dysbiosis

There have been differing reports on the impact of CF genotype on gut microbiota. A higher evenness and biodiversity have been reported in F508del homozygous children and adults when compared to those who were heterozygous or had two other CF causing mutations [73]. However, it should be noted that this study determined biodiversity using TTGE, which is a method similar to DGGE where band profiles are looked at [73]. This means findings cannot be equated to an alpha diversity score obtained using 16S pyrosequencing or MiSeq

sequencing. The reported changes in specific bacterial groups of interest, as measured by qPCR, were namely an increase of *E. coli* in F508del homozygous individuals and higher levels of *F. prausnitzii,* and *Bifidobacterium* in non-F508del individuals [73]. An increased relative abundance of non-bifidobacterial Actinobacteria in F508del-homozygous individuals was also reported in a study in 18 adults with CF [77]. Distinct differences in alpha-diversity and relative species abundance have also been demonstrated in faecal microbiota transplanted germ-free CF mice [45]. In contrast, Miragoli et al. reported no difference in gut microbiota composition between F508del homozygous and heterozygous genotypes in 30 children and young adults with CF [71]. Similarly, a study in adults with CF found no difference in species richness or microbial diversity when comparing those with more severe class I-III mutations to those with IV-VI mutations [83]. However, the authors reported compositional changes at a family and genus level, with increased relative abundance of *Enterococcaceae* and decreased *Ruminococcaceae*, typically indicative of lower diversity, in those with class I-III mutations compared to those with less severe mutations [83].

Exocrine Pancreatic Status and CF-related Gut Dysbiosis

Several studies investigated the difference between the gut microbiota in pancreatic insufficient (PI) and sufficient (PS) pwCF. A significantly lower diversity was reported in PI children with CF, compared to PS and HCs [68]. Another study identified a non-significant downward trend in microbial diversity, being highest in healthy controls and lowest in PI children with CF [62]. A further study in adults showed no significant difference in either species richness or diversity of gut microbiota [83]. Taxonomic changes have been reported. For example, a higher relative abundance of *Ruminococcus* in one study and *Oscillibacter* in another study in PS compared to PI individuals has been reported [68, 83].

Cystic Fibrosis-Related Liver Disease

A pilot study has investigated the impact of liver cirrhosis on the gut microbiota in pwCF [69]. The authors reported a decreased relative abundance of *Bacteroides* and increase of *Prevotella, Erysipelotrichaceae* and *Clostridium* in faecal samples from 11 participants with CF-related liver cirrhosis compared to age matched children and adults with CF but no CFRLD [69]. In an animal model, C57BL/6J *Cftr*$^{-/-}$ mice, homozygous for the S489X CFTR mutation, fed a high medium chain triglyceride (MCT) were found to develop cholangiopathy, which was absent in wild type mice [12]. The presence of cholangiopathy in the *Cftr* knockout mice was associated with an enrichment of *E. coli,* a greater degree of intestinal inflammation, increased intestinal permeability and decreased secondary bile acids when compared to wild type mice [12]. Furthermore, the presence of an overabundance of *E. coli* associated with cholangiopathy and intestinal inflammation was largely preventable by the administration of a chow diet (supplemented with polyethylene glycol [PEG]) [12].

Dietary Factors and CF-related Gut Dysbiosis

No significant difference in microbiota diversity was reported between breastfed infants with CF and those receiving formula feed [55, 56]. One study reported an increased relative abundance of *Lactococcus* in those solely formula-fed [56] while a second study identified no taxonomic changes when those breast fed were excluded from analysis [51].

No studies investigating the link between macronutrient intake and gut microbiota in pwCF were identified in this review. As outlined above, a CFTR knockout mouse model however found that a high MCT diet was associated with an increased relative abundance of *E. coli* when compared to both *CFTR*$^{-/-}$ mice on a chow and PEG diet and wild type mice [12]. Three studies did however investigate the relation between the gut microbiota and overall micronutrient intake [80], flavonoid [81] and vitamin D intake [79] respectively in pwCF. Li et al. [80, 81] investigated the association between micronutrient, flavonoid intake and the gut microbiota in 16 pwCF. The authors found that only beta-carotene was associated with weighted and unweighted UniFrac distances, a measure of beta-diversity (Table 1) [80].

Various associations between different taxa and micronutrients were reported, including beta-carotene positively correlating with *Clostridium* and *Gemellales* [80]. For flavonoid intake, only Gallocatechin, which is present in black tea, was found to be significantly associated with overall gut microbiota variations (presence or absence) in unweighted UniFrac analysis [81]. For weighted UniFrac analysis, variations in bacterial taxa were associated with certain flavonoids, of which only Gallocatechin correlated with specific bacterial taxa, including a positive correlation with *Actinomyces* and *Actinomycetaceae* and correlating negatively with *Coriobacteria* [81]. *Bacteroidia* and *Gammaproteobacteria* were enriched in adults with Vitamin D sufficiency and insufficiency respectively [79].

Medications and CF-related Gut Dysbiosis

While, in general, a higher prevalence of Proteobacteria has in general been associated with PPI use [85], the reported increased prevalence of Proteobacteria in infants with CF appears to be independent of PPI use [49]. No significant differences in diversity, phylum or family level differences have been reported in adults with CF taking PPIs [83]. However, genus-level changes have been detected, with a decreased relative abundance of *Dialister* in those on PPIs [83].

Multiple studies have investigated the impact of current/recent antibiotic exposure [50, 51, 56, 62], continuous antibiotics use and/or the cumulative effect of antibiotics on the gut microbiota in pwCF [50, 62, 80, 83]. Findings varied between studies. One study in children with CF, reported similar taxonomic profile differences between the CF and non-CF samples when they excluded CF samples taken up to 60 days post antibiotics [51]. Another study in adults found no significant difference in diversity in those taking continuous macrolide antibiotics compared to those who were not [83]. In contrast, a handful of studies found that children with CF who were taking antibiotics had a lower alpha-diversity [50, 56, 62], distinct beta-diversity clustering [56] and taxonomic changes, such as lower relative abundance of *Bifidobacterium*

[50, 56, 62] and *Bacteroides* [50, 57] and increased relative abundance of *Enterococcus* [50]. The use of continuous macrolide antibiotics in adults with CF was associated with significant decreases in the relative abundance of certain bacteria, including *Ruminococcaceae*, *Bifidobacterium* and *Akkermansia* [83].

Two studies found that aerosol antibiotics had no significant impact on the gut microbiota in children and young adults with CF [62, 69]. In contrast, Li et al. [80] reported inhaled, but not oral, antibiotics were associated with gut microbiota variation in adults with CF. With regards to cumulative intravenous (IV) antibiotic use in the last year, one study reported a negative correlation between the number of IV antibiotic courses and gut microbiota diversity [83]. The highest exposure of IV antibiotics was associated with the lowest Bacteroidetes and highest Firmicutes proportions [83].

Recently, there has been growing interest in the potential impact of CFTR modulators on gut dysbiosis in pwCF. A small study of 16 children and adults with CF reported no significant change in gut microbiota diversity, but a significant increase in the relative abundance of *Akkermansia,* after six months of ivacaftor (IVA) therapy [72]. A second study reported a significant increase in diversity in children and adults with CF but no significant compositional changes following IVA at either two, nine or 12 months of treatment [75]. Larger studies investigating the impact of the newer CFTR modulators, including elexacaftor/tezacaftor/ivacaftor are ongoing.

Consequences of CF Related Gut Dysbiosis

No studies investigating GI symptoms and gut microbiota in pwCF were identified. A few studies investigated the association between intestinal inflammation and the gut microbiota in pwCF. These studies predominantly measured intestinal inflammation using faecal calprotectin (FC). The levels of FC defining high intestinal inflammation varied between

studies, ranging from 50-250mg/g [58, 59]. Some studies found no relationship between FC and faecal microbial composition [49, 71]. Others found changes in microbial composition with FC levels, but the taxa identified varied between them. For example, a positive correlation between FC and relative abundance of *Streptococcus*, Firmicutes and *Staphylococcus* were reported [59]. Negative correlations in relative abundance with FC included *Bacteroides* [57], *F. prausnitzii* [59, 63] and *Bifidobacterium* [59].

There were no studies which directly measured markers of systemic or lung inflammation in pwCF. One mouse model investigated the impact of CFTR dysfunction on the adaptive immune response [45]. In CF specific-pathogen-free mice, there was a higher total cell count and larger number of T-cells in the mesenteric lymph nodes compared to non-CF mice, with no difference in T-cell count in the faecal microbiota transplant CF and non-CF mice [45]. Another study investigating the apical inoculation of Caco-2 cells with *Bacteroides,* found that it significantly reduced apical and basolateral IL-8 production [48].

A couple of studies found no significant correlation between gut microbiota and forced expiratory volume in one second (FEV_1) in children and young adults [56, 71]. Similarly, Coffey et al. [58] found no correlation between percentage predicted FEV_1 ($ppFEV_1$) and alpha diversity but did find $ppFEV_1$ positively correlated with the relative abundance of certain genera, including *Ruminococcaceae NK4A214* group, in children with CF. In adults with CF, those with severe lung disease ($ppFEV_1$ ≤40%) had significantly reduced alpha-diversity relative to those with mild or moderate disease [83]. The authors found that $ppFEV_1$ had the greatest correlation with alpha-diversity rather than any one bacterial population, with no significant phylum or family level changes found [83]. There were genus-level changes, including significantly higher relative abundance of *Roseburia* in those with mild lung disease ($ppFEV_1$ ≥70%) [83].

In a longitudinal cohort study of 13 infants with CF, a non-significant decrease in the relative abundance of *Bacteroides* and *Bifidobacterium* was reported prior to first pulmonary exacerbation [54]. In a separate study of 20 infants with CF, hospitalization for pulmonary exacerbation in the prior six months was not associated with significant species changes [56].

Discussion

To the best of our knowledge, this is the first systematic review on gut microbiota and cystic fibrosis. We explored the available evidence on the prevalence and impact of gut dysbiosis in people with CF. We found that despite different study designs, population characteristics and analytic techniques, characteristics of a CF specific gut microbiota were clear and transcended these differences. Cystic fibrosis related gut dysbiosis (CFRGD) remains an important clinical manifestation of this complex multisystem condition.

Pre-weaning, the gut microbiota diversity tends to be lower in healthy young infants, with a high abundance of *Bifidobacterium* [86, 87]. In contrast, a higher gut microbiota diversity was often seen in young infants with CF [48, 50]. A higher gut microbiota diversity has also been reported in formula fed infants, compared to those breastfed [86, 87], and this, in some instances, represents an outgrowth of less beneficial bacteria, such as *Clostridioides difficile* [87], *Streptococcus, Enterococcus* and *Veillonella* [86]. Once weaning commences, diversity increases rapidly and a high microbiota diversity becomes indicative of a healthy gut as it represents the build-up for various (anti-inflammatory) commensal species working together forming independent trophic networks enhancing the fermentative function of the gut [88-91], while a reduced diversity is associated with several inflammatory conditions, such as inflammatory bowel disease (IBD), type I diabetes and psoriatic arthritis [1, 92, 93]. The majority of studies included in this review identified a reduction in microbial diversity in children (post-weaning) and adults with CF and the presence of a gut microbiota which might be considered pro-inflammatory, when compared to healthy controls [57, 58, 68].

Cystic fibrosis results in an increase in pathobiont and pro-inflammatory associated gut bacteria and an overall reduction in bacteria which are linked to anti-inflammatory effects. This shifted microbial composition mirrors aspects of the compositions seen in extremely premature infants [94], inflammatory conditions such as IBD [95], asthma [96] and colorectal cancer (CRC) [97]. An increased relative abundance of bacteria typically associated with the small intestine is frequently found in pwCF, including *Lactobacillus, Clostridium, Streptococcus, Veillonella* and *Enterococcus* (e.g.) [48, 50, 62, 64, 68, 77, 78, 98, 99]. Perhaps an impaired intestinal barrier might be limiting strictly anaerobic colonic commensal bacteria by limiting the colonic oxygen concentration to a lesser degree.

Furthermore, the microbiota of pwCF is associated with an increased relative abundance of bacteria, known outside of CF, to produce immuno-stimulating LPS, such as *E. coli* and *Veillonella parvula* [20, 51, 53, 64]. In contrast, the relative abundance of bacteria associated with anti-inflammatory properties, such as *Bifidobacterium* and *Faecalibactrium prausnitzii,* were decreased in pwCF [24, 48, 50, 57, 62, 64, 71, 76, 78, 83, 100]. Low abundance of *Faecalibactrium prausnitzii* in CRC, a condition which has an increase prevalence in CF, has been associated with increased TNF and NF-KB expression, and reduced survival [101]. A reduction in the relative abundance of butyrate-producing bacteria in pwCF such as *Roseburia* [62, 64, 83, 102] and *Agathobacter rectalis* [47, 51, 57, 63, 71, 103] has also been reported.

Despite evidence for a reduction in SCFA-producing bacteria only two studies directly measuring SCFA levels in CF stool were identified. The first reported higher overall SCFA levels in children without CF [62] and the second study identified lower levels of acetate in adult CF faecal slurry [47]. In this latter study, which was an *in vitro* fermentation model seeded with a CF or HC stool slurry, the effects of prebiotic supplementation were investigated [47]. While CF faecal slurry was able to ferment the high amylose maize starch supplement

producing SCFAs in the process, though at a slightly more limited capability than HC slurries, in some of these samples it resulted in an increase of potentially pathogenic bacteria [47]. However, this *in vitro* model may not have been reflective of the CF gut as it used a fixed pH, and experiments were conducted on a single substrate [47]. Functional pathway analyses by Manor et al. [51] corroborate with the above findings, reporting enriched butyrate and propionate catabolism pathways and reduced fatty acid biosynthesis in pwCF. The study was undertaken in very young children (<3 years) and did not separate the results according to age, a factor which may have confounded the expected age-related development shifts in the gut microbiota [51]. This limits the ability to determine whether the combination of a low fibre diet, dysbiosis and CFTR dysfunction influences the levels and ratio of SCFAs in the CF gut and whether these changes have a profound long-term effect on innate and adaptive immunity, inflammatory and CRC risk, given that all of these are accentuated in CF.

Pathobiont gut bacterial species, such as *Faecalicatena gnavus* and *C. difficile* [62, 78] have an increased relative abundance in CF. The high carriage rates of *C. difficile* in patients with CF does not however translate into high rate of *C. difficile* diarrhoea [104]; a possible result of a low intestinal pH and the development of immuno-tolerance during early and constitutive bacterial exposure [23, 104]. Other uncommon and opportunistic pathogenic bacteria, such as *Clostridium hathewayi* [78], *Clostridium symbiosum* [47] and *Clostridium innocuum,* have also been reported in CF stool, although the clinical significance remains unclear [58].

The gut microbiota provides an important barrier against harmful pathogens through multiple complex interactions, including nutrient metabolism. In pwCF there is a tendency for an increase in amino acid degradation and a decrease in vitamin B metabolism pathways [58, 61, 77, 78]. The clinical consequences of these changes and the degree to which diet contributes to these shifts have yet to be determined [58, 78]. The microbiota in pwCF is also associated

with increased antibiotic resistance and enriched xenobiotic degradation pathways [48, 67, 82], processes which may influence treatment efficacy.

A link between different CF genotype and extent of gut dysbiosis has been reported, with more severe mutations leaning towards an increased relative abundance of pathobiont bacteria, such as *E. coli*, and a reduction in the relative abundance of anti-inflammatory bacteria, such as *F. prausnitzii* [62, 73]. This may simply reflect pancreatic status and disease severity. However, a study in a CF mouse model points to CFTR dysfunction exerting an independent influence on both the innate immune response and GI microbiome [45]. It could be theorised that the impaired mucus production in pwCF may be compounding gut dysbiosis, given that several of the species with an increased abundance in pwCF mirror those of extremely premature infants, who also likely have compromised intestinal mucus production [105].

The influence of pancreatic function on the gut microbiome in CF remains unclear, due in part to the small numbers of PS participants included in studies [78, 83]. Hypothetically, PI should result in more pronounced gut dysbiosis, due to alterations in nutrient availability, its association with more severe CFTR phenotypes, and worse clinical trajectory [106]. Future large-scale studies are needed to further characterise the relationship between pancreatic function and gut dysbiosis in CF.

While gut microbiota perturbances have been implicated in the pathogenesis of various liver disease pathologies, including non-alcoholic fatty liver disease, primary biliary cholangitis and cirrhosis, only two studies have investigating this in the context of CF, one in humans and one in mice [12, 69, 107]. In a CFTR knockout mouse model, complex interactions between the underlying CFTR dysfunction, diet, gut microbiota and liver disease have been observed [12]. The pilot study in children and adults with CF found differences between those

with CF-related liver cirrhosis and those without CFRLD [69], which mirror findings outside of CF, where higher levels of (oral) *Prevotella* and *Clostridium* (including *C. perfringens*) with liver cirrhosis have also been reported in faecal samples [108]. Given these findings, further research is warranted. Data were lacking in regard to whether CF-related diabetes further accentuates gut dysbiosis in pwCF, which was surprising in view of the link between type I and type II diabetes mellitus and gut dysbiosis [109, 110].

Aberrations in the gut microbiota have been linked with non CF GI motility [111], which is pertinent in view of the link between gut dysmotility and CF [112, 113]. While not initially included in this review, a recent pilot study investigating the impact of gut dysmotility and gut microbiota in pwCF [114], found that gut physiology and transit time significantly contributed to whole gut microbiota variance in a small cohort of adolescents and adults with CF and ten age-matched HCs [114]. This provides further tentative support for a complex interplay of factors driving CFRGD, with larger studies ongoing.

While evaluating the association between anthropometrics and gut microbiota was outside the scope of this review, prospective research is needed to characterise the impact of the rising levels of overweight and obese status and whether there is a concomitant shift to a more obesity associated gut microbiome in pwCF [115]. As lifespan and nutritional status improve, there is the growing need to understand the impact of diet on the gut microbiota and longer-term metabolic complications in pwCF.

In non-CF infants, breastfeeding significantly impacts the gut microbiota and is associated with a higher relative abundance of *Bifidobacterium* [4], while formula-feeding results in a higher prevalence of pathobiont bacteria, such as *C. difficile* and *Citrobacter* [116]. Intriguingly, similar differences between these feeding methods were not identified in CF infants [51, 53], a potential reflection of low breast feeding rates in infants with CF [117, 118].

As formula feeding has been linked to an increased risk of developing inflammatory conditions, such as asthma and allergic disease [119], the potential benefits of breast feeding in infants with CF needs further exploration. One small study identified a non-significant trend between an increase in time to first pulmonary exacerbation and breastfeeding in infants with CF [54].

The 'CF diet' has been linked to clinical outcomes in CF, with a high fat, high energy diet being advocated and taken up worldwide following the seminal work by Douglas Crozier, in Toronto [120, 121]. This high protein, high fat and, consequently, low fibre diet resulted in improved survival in the era before CFTR modulator therapy [8, 122]. However, outside of CF, this diet has been demonstrated to be detrimental for the gut microbiota and potentially could have a long-term negative impact as lifespan increases in pwCF [10, 11, 123]. There is, however, minimal direct evidence on the extent to which macronutrient intake is driving CFRGD in pwCF.

The studies evaluating micronutrient intakes need to be interpreted with caution owing to the small sample size and the risk of confounding factors [80, 81].Vitamin D is routinely supplemented in PI patients with CF and there is increasing evidence that deficiency can result in impaired gut barrier function and influence the innate immune response, associated with systemic autoimmune diseases [124]. Differences in the gut microbiota have been reported in vitamin D sufficient and insufficient individuals with CF, although these findings may reflect the study design and an imbalance between CFTR genotypes in both groups [79]. Given the significant improvements seen in nutritional status with CFTR modulators, dietary modification is needed, potentially including the Mediterranean diet, which is associated with a higher abundance of beneficial bacteria and SCFA production [125, 126].

Several studies have reported on the impact of probiotics on the CF the gut microbiota [57, 60, 70]. Results varied between studies as they studied different populations with

differences in regards to age, pancreatic status, the probiotics used and which gut microbiota analysis methods were employed [57, 60, 70]. Taking a broad view, trying to take the limitations of these studies into account, both Bruzzese et al. [57] and del Campo et al. [70] essentially report that administration of either *Lactobacillus rhamnosus* strain GG (LGG) [57] or *Lactobacillus reuteri* [70] resulted in a decrease in intestinal inflammation, a significant increase in *Bacteroides* counts and non-significant increase in *F. prausnitzii* with LGG treatment [57] and a reduction in Proteobacteria and increase in Firmicutes and *Bacteroides* with *L. reuteri* administration [70]. These changes point towards a shift from the more dysbiotic *Bacteroides*2 enterotype, characterized by elevated Proteobacteria and inflammation levels and reduced alpha diversity [127, 128], towards a composition that might be classified more as a *Bacteroides*1 enterotype-like composition. Probiotics supplementation in another study with *Lactobacillus rhamnosus* & *Bifidobacterium animalis*, in contrast, did not have a significant affect at a phylum or phylogenic level [60]. In a more recent study, not included in the initial selection of papers in this systematic review, the effect of LGG was similar to that reported by Bruzzese et al. [57] and del Campo et al. [70], with a shift being observed towards a less dysbiotic composition, a (non-significant) trend of increased alpha diversity and a *Bifidobacterium*-dominated gut microbiota, which in turn was associated with lower intestinal inflammation [129]. Disparities between these studies findings could have arisen from different molecular analysis techniques, varying participant characteristics and highly inter-individual changes in the gut microbiota, which was noted by three of these studies [60, 70, 129]. Details of the impact of probiotics on other clinical outcomes, such as intestinal inflammation and pulmonary exacerbation frequency, have been reported in a number of systematic reviews [130-133]. Data remains limited and further research is needed before probiotic administration can be formally recommended to treat gut dysbiosis in CF.

The burden of polypharmacy in people with CF can impact on quality of life. It is not surprising that some drugs result in gastrointestinal side effects and a negative impact on the gut microbiome. In the general population, PPIs have been linked with gut dysbiosis and increase in *E. coli* and *C. difficile* infection [14, 134]. Similar changes are not apparent in CF [49, 83], possibly because the presence of CF obscures this effect by inducing a similar kind or even more severe form of dysbiosis itself.

Antibiotics are the mainstay of CF treatment and, in the non-CF population, are associated with significant and long-lasting effects on the gut microbiota [13, 135] and the development of inflammation-associated conditions including Crohn's Disease [136], colon cancer [137] and asthma [138]. Their use in animal models has been shown to reduce SCFA levels [139] and increase bacterial translocation and inflammation [140]. In CF, antibiotics have been shown to decrease the relative abundance of commensal bacteria, such as *Bifidobacterium* and *Bacteroides*, and reduce alpha-diversity [50, 56, 57]. A negative correlation between IV antibiotic courses and gut microbiota diversity has also been reported [83]. Although it appears that primarily taxonomic differences in pwCF are present independent on antibiotic use [51, 69]. More detailed studies on the impact of the cumulative effect of antibiotics on the gut microbiota composition, functionality and SCFAs are required.

The introduction of CFTR modulators herald a new era for CF management. These small molecules are able to partially correct CFTR dysfunction and have the potential to normalise gut luminal conditions and improve the gut microbiota [72, 141]. The first modulator introduced into clinical practice was IVA and studies to date have reported both significant [75] and non-significant changes in bacterial diversity [72]. Confounding factors, such as a reduction in pulmonary exacerbations and antibiotic usage following IVA therapy may have contributed to these differing findings [75]. An increase in the relative abundance of *Akkermansia*, following IVA, has also been reported in 16 adults and children with CF [72].

Akkermansia muciniphila has been associated with anti-inflammatory effects [142]. However, from a microbial compositional point of view a high *Akkermansia* abundance is indicative of not being in a *Prevotella* rich composition (*Prevotella* enterotype), which might be considered neutral, but more importantly, is also indicative of not having a composition defined in some literature as the *Bacteroides*2 enterotype, which is relatively rich in *Enterobacteriaceae*, Fusobacteria and *Faecalicatena gnavus*, has a low diversity, and can generally be regarded as dysbiotic and not associated with good health [127, 143, 144]. Overall, the evidence to date suggest that IVA does not completely reverse CFRGD but larger studies which include the impact of newer triple combinations of CFTR modulators are needed. Some of the potential benefits of CFTR modulators may be subdued by the deleterious effects of factors such as diet, pancreatic malabsorption and chronic GI dysfunction. A high fat intake is known to drive gut dysbiosis and pwCFs' fat intake has been shown to significantly increase when taking IVA [12, 145].

Cystic fibrosis is associated with increased intestinal inflammation, generating a milieu favouring the growth of pathobiont and pro-inflammatory bacteria, such as *E. coli* [95]. There does not seem to be a clear relationship between FC and alteration in CF microbiota, although significant inter-subject variation in FC levels have been reported [49, 53, 71]. In general, FC levels positively correlated with the relative abundance of pro-inflammatory bacteria, such as *Streptococcus* [59] and negatively correlated with the relative abundance of anti-inflammatory bacteria, such as *Bifidobacterium* and *F. prausnitzii* [57, 59]. More studies that characterise the relationship between intestinal inflammation and CFRGD are needed.

The role of the gut-lung axis has long been postulated to alter the immune and inflammatory response in diseases, such as asthma and CF. One hypothesis is that CFRGD drives excess systemic and lung inflammation, as reported in other inflammatory conditions

[92, 146]. However, there is a lack of studies directly measuring the link between gut dysbiosis in pwCF and markers of systemic or lung inflammation in pwCF.

Disease severity, as reflected by lung function and pulmonary exacerbations, could accentuate gut dysbiosis in CF, due to the many negative influences, such as increased antibiotic exposure and could be linked directly due to a shared genetic aetiology. In adults with CF, severe lung disease is associated with reduced faecal alpha-diversity [83] while in children and young adults no significant correlation between gut microbiota and lung function was reported [56, 58, 71]. Similarly, there was little convincing evidence on any relationship between the gut microbiota and pulmonary exacerbations in pwCF.

No studies identified in this review reported on the gut microbiota and GI symptom, despite the frequency of GI symptoms in pwCF [147] and the fact that gut dysbiosis is likely to be involved in the pathogenesis of symptoms in other GI conditions [148, 149]. However, studies are ongoing which are likely to determine the role of dysbiosis in GI symptomology.

Different outcomes and conclusions between studies are likely to reflect biological and technical differences, which will have influenced the observed different taxonomic shifts. For instance, there are known geographical and inter-individual variations in the gut microbiota [150, 151]. Studies were often small with differing cohort demographics, making direct comparison more challenging. Of extreme importance is the age effect in regards to *Bifidobacterium* and age/weaning. The gut microbiome develops very rapidly during weaning, though less so in pwCF, making cohorts that combine children from very young ages (<1y) with older children/adults less reliable [152]. In addition, technical variations in sample collection, processing, length of storage and analysis will all influence results. For example, as *Lachnospiraceae* are strictly anaerobic, the longer the stool samples are exposed to oxygen, the greater degree of bacterial death which in turn influences findings [153]. Another example

is that the abundance of *Bacteroides* detection decreases as the length of -80^{0}C storage increases [154]. Comparisons are furthermore confounded by the fact that a variety of different meta-genomic analysis methods, selected primer regions and databases are employed [155, 156]. Excluding geographical and inter-individual variation, comparisons between different studies can only be reliably made by comparing the direction of ratios of potentially important microbial groups between cases and controls. Together with the heterogeneity and nature of the studies identified, this results in a high risk of confounding factors, which need to be considered when drawing conclusions. They also highlight the need for large-scale studies with more harmonious methodology in this area.

Conclusions

Cystic fibrosis related gut dysbiosis is common and it is characterised by an altered bacterial microbiota diversity. The divergence appears to represent a pro-inflammatory bacterial milieu, with alterations in the ratio of immune-stimulating and immune-silencing bacteria. However, little direct evidence links the impact of gut dysbiosis with systemic inflammation and lung health. It is currently not clear if CFTR modulators will be able to reverse CFRGD and further research is needed to identify key drivers which will improve gut health and reduce inflammation. Given the complex aetiology of CFRGD in pwCF, the extent to which dietary advice could achieve specific gut microbiota shifts warrants exploration.

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Conflicts of Interest

None

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