

# Celiac disease and human gut microbiota – how can we study the composition of microorganisms?

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## Abstract

Celiac disease is an autoimmune disorder induced by consumption of gluten protein present in foods such as wheat and rye. In recent years there has been increasing evidence that changes in composition of gut microbiota may play a significant role in the pathogenesis of celiac disease. Multiple methods of bacterial identification may be used to find microbiota changes characteristic for celiac disease, and the latest methods such as next generation sequencing offer new possibilities of detecting previously unknown bacterial groups that may play a role in the occurrence of celiac disease. This review focuses on multiple methods of identifying bacterial gut microbiome and presents results of recent studies exploring the link between gut microbiota composition and celiac disease.

## Introduction

The term “gut microbiota” refers to the collection of bacteria, archaea, viruses (mainly bacteriophages), and Eukarya colonising the gastrointestinal tract of humans. However the microbiome refers to the collection of genomes from all the microorganisms in the given environment (in this case, the intestine) [1]. Human gut is populated with up to 100 trillion microbes [2], with about 9,879,896 microbial genes [3] and 4644 different species of prokaryotes [4] of gut microbiota currently identified. Most (93.5%) bacteria present there are members of 4 bacterial phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* [5]. The composition of microbiota is shaped by both host and environment. On host's side, the intestinal immune system minimises adverse effects of gut microorganisms on health and maintains homeostasis by limiting tissue invasion using a hierarchy of immunological barriers [6]. Several pro-inflammatory and anti-inflammatory processes are maintained in balance, which ensures normal

physiological function of the gastrointestinal tract. The disruption of this balance and homeostasis may lead to disease [7]. On the other hand, number of factors such as age [8], geographical location and ethnicity [9], smoking [10], and diet [11, 12] may affect the composition of gut microbiota. This enormous system has a complicated but significant relationship with human health. While necessary for proper functioning of the human organism, providing benefits such as break-down of dietary elements into bioactive food components, vitamin synthesis, prevention of invasion of gastrointestinal tract surfaces by hostile microorganism, anti-inflammatory effect on gut [13], and possibly regulating cognitive abilities [14], there is evidence that disruption of microbial gut composition and/or function may lead to disease. Gut dysbiosis has been linked to the development of obesity [15], irritable bowel syndrome [16], psychiatric disorders [17], and celiac disease.

Celiac disease (CD) is an autoimmune disorder that affects genetically predisposed individuals. The disease starts in susceptible patients after ingestion of glu-

ten – a mixture of proteins presents in cereals. After consumption, the peptides pass through the epithelial barrier of the intestine and react with antigen-presenting cells in the lamina propria. The immune response to gluten in celiac patients promotes an inflammatory reaction, with infiltration of the epithelium and lamina propria by chronic inflammatory cells and villous atrophy. Clinical manifestations of celiac disease include diarrhoea or constipation, abdominal distension, vomiting, and failure to thrive in children [18]. Apart from genetic factors and gluten exposure, there are other environmental factors that may play a role in the development of celiac disease, including gut microbiota dysbiosis [19]. Currently, the only widely used treatment of celiac disease is a lifelong gluten-free diet (GFD). Adherence to GFD ensures clinical improvement within the first few weeks and recovery of the mucosal damage in 1–2 years [20]. Another potential treatment option is the use of probiotics. As has been shown, bacteria of the gut microbiome are involved in gluten metabolism [21], and dysbiosis may lead to dysfunction in the gut barrier [22]. Thus, correcting effects of dysbiosis with use of probiotics, mainly *Bifidobacterium* and *Lactobacillus*, may help reduce the symptoms of celiac disease and correct dysbiosis [23]. Other possible interventions

that are still being developed include the use of gluten-degrading enzymes, regulation of inflammatory mediators that play role in pathogenesis of CD, and therapies enhancing the function of the gut barrier [24].

There are several methods of exploring the composition of human gut microbiota, which might help to discover a link between certain gut bacteria and celiac disease. Each of these methods offer several advantages and disadvantages. In addition to the method of gut bacteria identification, other factors may play a role in the composition of intestinal microbiota, such as status of disease – whether patients were treated using a gluten-free diet (tCD) or were untreated (uCD), and the age of study participants (children or adults). Moreover, the sampling method may also impact the results. While faecal samples are often used as proxies for studies of gut microbiota, there may be significant differences between microbial composition in faeces and the actual composition of microbiota in the gut. Whilst invasive, use of biopsies is often more representative of gut microbiome contents [25]. This review includes studies using both faecal and biopsy samples.

This paper presents the characteristics of the methods used in microbiota and microbiome profiling, as well as their strengths and weaknesses, in patients with

**Table I.** Comparison of techniques used in identification of gut microbiota

Technique	Strengths	Limitations
Next generation sequencing – targeted amplicon sequencing	<ul style="list-style-type: none"> <li>– Fast (dependent on platform)</li> <li>– Relatively simple, with standardised data analysis and multiple reference databases</li> </ul>	<ul style="list-style-type: none"> <li>– Often restricted to genus-level, inability to differentiate at species-level</li> <li>– Restricted to relative abundance</li> <li>– Possible errors during DNA extraction and PCR quantification</li> <li>– Higher cost</li> </ul>
Next generation sequencing – shotgun metagenomic sequencing	<ul style="list-style-type: none"> <li>– Highly accurate</li> <li>– High reproducibility</li> <li>– Allows identification at species-level</li> </ul>	<ul style="list-style-type: none"> <li>– Higher costs</li> <li>– Complex informatic analysis</li> <li>– Higher risk of host contamination</li> </ul>
Next generation sequencing – shallow metagenomic sequencing	<ul style="list-style-type: none"> <li>– Highly accurate</li> <li>– High reproducibility</li> <li>– Less costly and complex in informatic analysis than shotgun metagenomic sequencing (“deep sequencing”)</li> </ul>	<ul style="list-style-type: none"> <li>– Often restricted to genus-level, inability to differentiate at species-level</li> <li>– Higher risk of host contamination</li> </ul>
Real-time qPCR	<ul style="list-style-type: none"> <li>– Fast</li> <li>– Inexpensive</li> <li>– Ability to determine absolute abundance of taxa</li> </ul>	<ul style="list-style-type: none"> <li>– Unable to detect unknown species</li> <li>– Possible errors during DNA extraction and PCR quantification</li> </ul>
FISH/flow cytometry	<ul style="list-style-type: none"> <li>– Fast</li> <li>– Inexpensive</li> <li>– Avoids PCR errors</li> </ul>	<ul style="list-style-type: none"> <li>– Unable to detect unknown species</li> <li>– Lower sensitivity</li> </ul>
DGGE/TGGE	<ul style="list-style-type: none"> <li>– Inexpensive</li> <li>– Ability to extract bands for further analysis</li> </ul>	<ul style="list-style-type: none"> <li>– Problems with reproducibility</li> <li>– Problems with identifying single populations</li> <li>– Possible errors during DNA extraction and PCR quantification</li> </ul>
Culturomics	<ul style="list-style-type: none"> <li>– Inexpensive</li> <li>– Ability to detect populations with low cell counts</li> </ul>	<ul style="list-style-type: none"> <li>– Slow</li> <li>– Many bacterial species are still unculturable</li> </ul>

celiac disease. The compared methods are presented in Table I.

## Sequencing methods

### Next Generation Sequencing (NGS)

Since their introduction in 2005 [26] next generation sequencing (NGS) technologies have revolutionised genomics and have found many uses in fields such as clinical microbiology [27], oncology [28], and clinical genetics [29]. NGS allows sequencing of entire genomes cheaper and more rapidly than Sanger sequencing methods. Thousands to millions of sequencing reactions are processed in parallel at the same time, instead of hundreds, and the sequencing output is detected directly without need for electrophoresis [30]. First technology that was considered as NGS was pyrosequencing, introduced in 2005 by 454 Life Sciences. In the following years, many new systems for NGS were developed, and currently there are multiple NGS platforms available on the market (e.g. Illumina, IonTorrent, PacBio) [31]. Based on different methods of DNA immobilisation on a solid substrate, 3 NGS high-throughput sequencing technologies were commercialised: high-throughput pyrosequencing on beads, sequencing by ligation on beads, and sequencing by synthesis on a glass substrate [32].

The NGS method consists of 3 steps: template preparation, sequencing with imaging, and data analysis [33]. During template preparation the library of nucleic acids is constructed and adapted to used sequencing system [34]. DNA or RNA is fragmented to the desired length and converted to double-stranded DNA. The fragmentation may be done by either enzymatic methods or physical methods, such as nebulisation or sonication. Although with minimal differences, all these methods offer similar performance [35]. Then, oligonucleotide adapters are attached at the ends of used fragments. Adapters are platform-specific sequences, which allow recognition of sequenced fragments using dedicated sample-indexing oligonucleotides by the NGS platform used in sequencing. The next step includes quantitation of the prepared library.

The sequencing process includes 2 core elements: clonal amplification and actual sequencing. During clonal amplification DNA fragments bind to the solid phase, and after anchoring they are amplified by polymerase chain reaction (PCR) [36]. Methods of sequencing include sequencing by hybridisation and sequencing by synthesis – SBS (Ion Torrent, Illumina, 454 Life Sciences pyrosequencing, PacBio). Sequencing by synthesis methods do not generally use dideoxy terminators used in original Sanger sequencing and rely on shorter reads of about 300–500 bases. In most SBS platforms, DNA molecules are distributed to millions of separate wells

or chambers. The DNA molecules are then amplified and subjected to DNA synthesis reactions, in which, depending on the used platform, labelled nucleotides or chemical reactions of nucleotides are imaged or otherwise detected [37]. Once sequencing is completed, a significant amount of raw sequence data produced must be analysed. A variety of software tools are available for analysing NGS data [38].

During microbiome analysis 2 sequencing strategies may be applied: 16S rRNA amplicon sequencing or entire genome metagenomic sequencing [39].

### NGS – Targeted amplicon sequencing

The amplicon sequencing relies on analysis of DNA fragments amplified by PCR (amplicons). The fragments usually targeted are ribosomal 16S rRNA genes, albeit ribosomal 23 rRNA genes, the 16S-23S rRNA gene internal transcribed sequences, genes such as *rpoB* gene  $\beta$ -subunit of RNA polymerase, the *gyrB* gene encoding the  $\beta$ -subunit of DNA gyrase or the *groEL* gene encoding the  $\beta$ -subunit of DNA gyrase may be used as targets for identification using conserved sequences because those genes are found in virtually all bacteria [40]. The 16S rRNA gene is about 1550 bp long and is composed of 9 hypervariable regions (V1–V9) that are interspersed throughout highly conserved sequences.

Amplicon sequencing is relatively simple, inexpensive, and fast. In addition, the workflow of data analysis for amplicon sequencing is mostly standardised [41]. The method does not rely on whether the bacteria in the sample are culturable or not, the relative abundance of total bacteria in the sample can be determined, and hundreds of samples may be sequenced simultaneously [42]. Additionally, 16S rRNA amplicon sequencing has the advantage of massive amounts of publicly accessible sequence information in multiple reference databases [43].

However, the NGS 16S rRNA targeted amplicon sequencing method has many potential problems. The 16S amplicon method is limited to only a single region of the bacterial genome, and the method identifies fewer bacterial species per read compared to whole genome shotgun sequencing [44]. Due to 16S rRNA having only a housekeeping gene, that often only indicates phylogenetic divergence, sequencing cannot give information about the biological function, metabolic potential, or activity of microbial community. Additionally, due to limitations of the sequencer, the total microbial abundance is not reflected, and only relative abundance is presented. Total bacterial abundance requires a quantitative technique such as qPCR and cannot be obtained by 16S rRNA sequencing alone. The 16S method also cannot distinguish between dead and alive microbes

[45]. A complete and high-quality reference database must be used to ensure high accuracy of taxonomic identification. Partial 16S rRNA sequencing is also often restricted to genus-level taxonomic classification because it lacks the discriminatory power to differentiate prokaryotes at the species level. Other potential issues relate to sample collection and DNA extraction, PCR amplification, and bioinformatic analysis [43].

### **NGS – Shotgun metagenomic sequencing**

The shotgun metagenomic sequencing method relies on untargeted sequencing of whole DNA available from a sample. The genetic material is divided into small fragments, which are independently sequenced. This results in sequence reads that align to various locations of the bacterial genome, not only selected sites (e.g. 16S rRNA) as in amplicon sequencing [46].

This approach to sequencing offers significant advantages. The sequencing of the entire genome not only provides data for taxonomic identification, but also for biological functions encoded in the genome. Compared to 16S rRNA amplicon sequencing, shotgun metagenomic sequencing identifies approximately 2 times more species and may provide more accurate identification on species level [44]. Additionally, analysing the whole genome and not only selected genes, characteristic to prokaryotes, allows the detection of other domains such as fungi or parasites and viruses. On the other hand, the disadvantages of whole genome shotgun sequencing include its higher cost and higher risk of host contamination than in amplicon sequencing [47]. Furthermore, metagenomic data is relatively large and complex, which may present more computational problems in informatic analysis [39].

Because the main disadvantage of shotgun metagenomic sequencing is its high cost, due to large number of analysed sequence reads, an alternative method of shallow shotgun sequencing may be used. Compared to whole-metagenome shotgun sequencing (“deep shotgun sequencing”), shallow shotgun sequencing offers depth of far fewer reads – about 2 to 5 million reads per sample, compared to more than 10 million in deep shotgun sequencing – while providing nearly the same accuracy at species-level identification as deep shotgun sequencing. In addition, shallow shotgun sequencing seems to be better at classification at species level and with higher reproducibility than 16S amplicon sequencing. However, similarly to whole-metagenome shotgun sequencing, shallow sequencing is susceptible to contamination with host DNA. Also, shallow sequencing method cannot provide strain-level microbiome information and relies on species-level data

being present in the reference database, unlike deep sequencing [48, 49].

### **Other methods of identifying microorganisms**

#### **Real time qPCR**

Real time PCR (also known as quantitative PCR – qPCR) is method of quantification of genetic material, by utilising PCR amplification of DNA with fluorescence markers, which bind during amplification reaction and emit fluorescence lighting, which might be detected and analysed. These markers are either DNA dyes, which enable both specific and non-specific detection of amplified products, or fluorescently labelled oligonucleotide probes, which detect only specific PCR products [50]. The intensity of fluorescence is measured after each cycle. The increase of fluorescence intensity reflects the amount of DNA amplicons in a sample at a given time. The point called the quantification cycle (C<sub>q</sub>) refers to the moment at which fluorescence is distinguishable from the background. The C<sub>q</sub> value corresponds proportionally to the starting number of DNA molecules present in the sample and allows the determination of the absolute quantity of target DNA [51].

qPCR is an accessible, simple, and cost-effective method of bacteria identification. Due to quantitative character of the method, the qPCR allows determination of absolute, not only relative, abundances of individual taxa or all taxa of community at once if combined with NGS 16S rRNA gene amplicon sequencing [52]. There are, however, some disadvantages in using real time qPCR. This method cannot be used to detect unknown species due to the inability to design primers and probes [53]. Similarly to NGS methods, errors may occur during DNA extraction and PCR quantification of genetic material used in sequencing. Additionally, in qPCR there are 2 reporter systems commonly used: hybridisation probes and intercalating dyes, and use of each of them presents with potential problems. Intercalating dyes bind to all amplicons non-specifically, so their usage needs additional analyses (such as amplicon melting curve analysis) to confirm that only targeted genes were quantified. Hybridisation probes are designed to bind only to the conserved site on the target gene, to ensure quantification of the target gene. However, such conserved sites might not exist in the case of potentially unknown bacteria, so the probe might bind unequally to genes of all members and produce biased results [52].

#### **FISH/FCM**

FISH/flow cytometry is a method combining fluorescence in situ hybridisation (FISH) and flow cytometry (FCM). The FISH method uses fluorescently labelled

probes that hybridise to target complementary sequences in intact cells. A typical oligonucleotide probe is between 15 and 30 base pairs long, with directly bounded fluorescent dye. The FISH method comprises 5 steps: specimen fixation, sample preparation, hybridisation with fluorescent marked probes for detecting the respective target sequences, removal of unbound probes, and visualisation and documentation of results [54]. Fixation is responsible for preservation of cellular morphology in the case of tissue staining and for ensuring optimal retention of target sequences. Various fixatives are used, with paraformaldehyde and methanol being frequently used. In the case of Gram-positive cells, spore-forming bacteria, and mycobacteria, enzymatic permeabilisation of the cellular wall may be necessary to ensure reliable FISH staining. After preparation, hybridisation occurs. Numerous factors affect hybridisation process, including the GC-pair content of the probe and the length of the probe. Most FISH probes can be designed using specially adapted software tools and tested for specificity and binding conditions [55]. Afterwards, the results of hybridisation must be documented, either through conventional microscopy or flow cytometry. In the FISH/flow cytometry method, flow cytometry is used instead of conventional microscopy. This allows analysis of a higher number of cells simultaneously and with higher throughput [56].

Advantages of FISH/flow cytometry method include short time-to-result (60–90 min), relatively little difficulty of method and ability to identify bacteria at genus and species levels [50]. Combining the method with flow cytometry allows automated, quantitative analysis of bacteria. Additionally, because the method does not require use of PCR, a number of biases related to use of PCR are avoided [53]. However, FISH/flow cytometry presents many significant disadvantages. Because the method depends on oligonucleotide probes, it is not possible to characterise unknown, uncultured species. In the case of some bacteria and archaea, not every cell may be permeabilised using standard fixation protocols, although there are methods of fixation-free FISH [57].

### DGGE/TGGE

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are methods of analysis of PCR products utilising separation of DNA fragments. The basis of those methods is a decrease of electrophoretic mobility of partially melted double-strained DNA molecules in polyacrylamide gel. Once melting temperature  $T_m$  is reached in the domain with lowest value of  $T_m$ , the migration of molecules in the gel halts. Different DNA sequence variations have different melting temperatures, especially sequences

rich in GC pairs, which have higher  $T_m$ . Melting of double-strained DNA is achieved either by increasing the linear gradient of DNA chemical denaturants – mixture of urea and formamide (DGGE) – or by linear gradient of temperature (TGGE) [58].

DGGE and TGGE are low-cost methods of bacterial identification. The fingerprint bands received can be extracted from DGGE and applied for further analysis [59]. However, DGGE and TGGE methods face several limitations. There are problems with achieving reproducible results and difficulty with analysis of complex communities, because numerous populations in relatively equal proportions produce a smear of bands, which is hard to analyse and identify individual populations. Often single bands can represent multiple populations, and conversely a single bacterial population may be represented by multiple bands [60].

### Culture methods – “culturomics”

In recent years, metagenomic methods of studying human microbiome have mostly replaced culture-dependent methods. However, metagenomics may not provide the full picture of human microbiome, due to its limitations. For example, metagenomics suffers from difficulties in detecting microorganisms present at very low cell numbers due to trace amounts of reads compared to the entire pool obtained. Thus, usage of culturomics may address the drawbacks of metagenomics and help to fill the gaps in our knowledge of human microbiome and its effect on human health. Culturomics is defined as a high-throughput culturing approach, which uses multiple culture conditions and integrates MALDI-TOF mass spectrometry and 16S rRNA sequencing for accurate identification of cultured bacterial species [61].

A study using metagenomics estimated that at that time about 80% of detected bacterial species were not yet cultivated [62]. Indeed, while many species still are not cultivable, application of multiple culture conditions and different techniques allowed culturing of multiple species that were previously considered uncultivable [63, 64]. The usage of multiple culture conditions allows isolation of a number of bacterial species, with recent studies hinting that blood culture bottle with rumen fluid and sheep blood in anaerobic conditions may be best profitable while considering the number of species detected [65]. The presence of anaerobic conditions is especially important, because anaerobes are dominant members of human gut microbiota. Interestingly, in recent years there have been successful attempts to cultivate anaerobic bacteria, including strictly anaerobic, in aerobic conditions with use of Schaedler agar supplemented with glutathione and ascorbic acid [66]. Identification of cultured species may then be done

by use of MALDI-TOF mass spectrometry, a method of identification based on peptide spectra obtained by matrix-assisted laser desorption ionisation time-of-flight. Such identification is relatively cost-effective, accurate, and efficient [67]. Alternatively, 16S rRNA sequencing for the identification of bacterial species may be used.

Culturomics offer many advantages in the field of studying human gut microbiota, including the possibility of detecting minority populations and an increase of known bacterial species. Finally, pure culture is a crucial step in exploring bacterial physiology and characterising the relative roles of these microorganisms in host health and disease [65].

## Studies of microbiota in celiac disease

### NGS – amplicon sequencing

Among studies that analysed  $\alpha$ -diversity and  $\beta$ -diversity of intestinal microbiota, only one study using next generation sequencing included in this review found significant differences in both  $\alpha$ - and  $\beta$ -diversity between healthy controls and celiac disease patients in faecal samples (Singh *et al.*) [68].  $\alpha$ -diversity refers to the diversity present within each single sample, and  $\beta$ -diversity refers to the extent of differentiation between pairs of samples [69].

The study exploring effect of *Bifidobacterium breve* on the intestinal microbiota in children observed that at baseline, phyla of *Firmicutes* and *Actinobacteria* were relatively less abundant and phylum of *Bacteroidetes* were relatively more abundant in CD than in healthy controls. The study also noted a decreased *Firmicutes*/*Bacteroidetes* ratio in CD compared to healthy children (HC). Selected microbial groups were further analysed using qPCR, which allowed for absolute quantification. Total *Lactobacillus* spp. group and *Enterobacteria* group were significantly lower in CD children, while total *Bacteroides fragilis* were significantly higher [70]. Members of the genus *Lactobacillus* are one of the most important bacteria with probiotic potential, with impacts on human health, including promotion of anti-inflammatory effects by inhibition of production of pro-inflammatory cytokines, including IL-6 and IL-8, upregulating the expression of TLR-2/TLR-6 heterodimer, a receptor that act as an inflammatory intracellular signalling network, and restoring the gastrointestinal barrier function directly or indirectly [71].

A study analysing microbiota of both celiac disease and diabetes mellitus type 1 patients showed no difference in bacterial composition at phylum or family levels between CD and HC. Relative abundance of *Shigella*/*E. coli*, *Ruminococcus*, *Alistipes*, *Oscillospira*, *Prevotella*, and *Clostridium* were significantly higher and *Parabacteroides*, *Dialister*, and *Bacteroides* were decreased

in the CD group compared to healthy controls [68]. *Prevotella* has been shown to increase susceptibility to intestinal mucosa inflammation in mice models, although no study confirming similar effect in humans have been performed [72]. In comparison to other diseases associated with gut dysbiosis, an increase of certain *Ruminococcus* bacteria has previously been linked to Crohn's disease, due to production of unique pro-inflammatory glucorhamnan polysaccharide, which was shown to potently induce TNF- $\alpha$  cytokine secretion [73]. Similarly, a decrease in the abundance of *Oscillospira* has been associated with Crohn's disease [74].

Zafeiropoulou *et al.* noted no difference in  $\alpha$  diversity between celiac children, both treated (tCD) and untreated (uCD), and a healthy control group. However,  $\beta$  diversity analysis confirmed that the microbiota structure of tCD children differed from other groups (HC and uCD). The study found differences in abundance in multiple operational taxonomic units (OTUs). About 31 OTUs were significantly lower in the uCD group compared to HC, while 29 OTUs differed significantly between tCD and HC. Of those 29, 13 OTUs were more abundant in tCD, with 10 being significantly higher, and 16 OTUs had lower relative abundance in tCD, with 3 being significantly lower. Eleven OTUs were significantly lower in celiac disease group (tCD + uCD) compared to HC. The 2 most influential OTUs were OTU\_53 *Clostridium sensu stricto* 1 followed by OTU\_143 *Ruminococcus*. Despite these differences there were no differences in profound dysbiosis at onset of celiac disease in the paediatric population compared to HC [75].

The study concerning the correlation between gut microbiota and serum TNF- $\alpha$  (Primec *et al.*) found significant positive correlation between TNF- $\alpha$  and *Verrucomicrobia*, *Parcubacteria*, and some unknown phyla of bacteria and archaea using NGS sequencing of genetic material from faecal samples of CD children [76]. A study by Sample *et al.* found that in faecal samples genera *Alistipes* and *Bacteroides* were significantly more enriched in uCD than HC [77].

Interestingly, some studies hint that, before onset of the disease, there may be specific microbiota markers in children at risk of celiac disease, which differs from healthy children. However, others showed no such difference in microbiota composition. Girdhar *et al.* found that, while no differences among phylum or genus levels were found by relative abundance analyses, significant differences between children who progressed to celiac disease and healthy controls were noted in amplicon sequence variants (ASV) obtained by sample inference using the DADA2 software package. ASVs enriched in children who later developed CD include *Dialister*, *Gemmiger*, *Ruminococcaceae*, *Roseburia*, *Dialister propi-*

*onicifaciens*, and *Clostridium sensu stricto* [78]. Millettich *et al.* showed that 7 genera (*Anaeroglobus*, *Barnesiella*, *Candidatus Soleaferrea*, *Eubacterium*, *Monoglobus*, *Senegalimassilia*, and *Oscillospiraceae* UCG.002) were more abundant in healthy infants than in infants who later developed celiac disease. Additionally, 3 genera (*Erysipelatoclostridium*, *Haemophilus*, and *Lachnospiraceae* NK4A136 group) were more prevalent in the future celiac disease group [79].

Another study (Olivares *et al.*) discovered that at 4 months of age, infants who later developed CD harboured a significantly higher proportion of *Firmicutes* in comparison to children who did not develop the disease. This increase of *Firmicutes* phylum was accompanied by a proportional reduction in *Proteobacteria* and *Bacteroidetes*, although the differences in these 2 phyla were not significant. However, between 4 and 6 months, *Firmicutes* showed a significant increase in healthy children, while no differences were detected in children who later developed CD. This resulted in no difference at phylum level between groups at 6 months. The HC group also showed an increase of microbial richness and diversity during the study period, while a similar increase did not occur in the CD group. Further analysis also showed an association between increased abundance of *Bifidobacterium longum* in the HC group and *Bifidobacterium breve* and *Enterococcus* spp. in the CD group [80]. In contrast, Rintala *et al.* did not find any statistically significant differences in microbiota in infants who later developed celiac disease [81]. Constante *et al.* noted significant differences in microbiota composition depending on sampling location as the key determinant. Samples of the first portion of the duodenum showed an increase of *Escherichia* and a decrease of *Dolosigranulum*, *Phenylobacterium*, *Acidovorax*, and *Moraxella* genera in CD patients compared to healthy controls. Samples from the second portion of the duodenum showed an increase of genus UCG.001 (*Prevotellaceae*) and a decrease of *Methylobacterium*, *Staphylococcus*, *Bacillus*, *Sellimonas*, *Bradyrhizobium*, *Delftia*, and *Moraxella* genera in CD patients, and samples from the third portion of the duodenum were characterised by increased *Neisseria* and *Peptostreptococcus* and decreased *Methylobacterium*, *Acinetobacter*, *Leuconostoc*, and *Phenylobacterium* [82]. Multiple *E. coli* strains have been linked to both Crohn's disease and ulcerative colitis, with multiple proposed mechanisms of induction of proinflammatory responses that may play a role in the pathogenesis of those diseases [83].

In a study (Panelli *et al.*) analysing the composition of microbiota using both duodenal mucosal and faecal samples, a decrease of *Firmicutes* and *Actinobacteria* phyla and *Streptococcus* genus, and an increase of *Pro-*

*teobacteria* phyla and *Neisseria* genus were observed in mucosal samples of CD patients compared to HC. However, no such differences were noted using stool samples. In active celiac disease patients' mucosal samples, an abundance of *Bacteroidetes* were decreased, while treated celiac disease showed an increase, compared to HC. Stool samples showed an increase of *Blautia*, *Coprococcus*, *Roseburia* spp. genera and *Bifidobacterium longum* in uCD and an increase of *Veillonella* spp., *Haemophilus* spp., *Faecalibacterium prausnitzii*, and *Veillonella dispar* in tCD [84].

A study by Nobel *et al.* concerning the effect of gluten exposure on gut microbiome in patients with celiac disease found increased abundance of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* after 14-day gluten exposure in celiac disease patients. No difference in  $\alpha$ -diversity was noted, while  $\beta$ -diversity was significantly different between CD patients and HC [85]. In contrast, Nylund *et al.* noted no significant difference in microbiota richness or diversity between the celiac disease group and healthy controls. In this study, abundance of only *Bifidobacterium* tended to be lower in CD than in HC, although this difference was only marginally significant ( $p = 0.067$ ) [86].

D'Argenio *et al.* observed greater abundance of *Betaproteobacteria* class, the *Neisseriales* order, the *Neisseriaceae* family, and the *Neisseria* genus in uCD compared to tCD and HC. The study also found that the *Neisseria flavescens* strains found in celiac patients may induce a pro-inflammatory immune response [87].

The study by Shi *et al.* regarding gut microbiota composition in Northwest China showed significant differences in bacterial communities between healthy controls and celiac disease patients. The abundance of *Proteobacteria* was higher in celiac disease patients, and at genus level there were 9 bacteria groups with differences between CD and HC. Abundance of *Lactobacillus*, *Veillonella*, *Allisonella*, and *Streptococcus* were significantly increased in the CD group, while groups *Ruminococcus*, *Faecalibacterium*, *Blautia*, *Gemmiger*, and *Anaerostipes* were decreased significantly [88].

Palmieri *et al.* found that the abundance of the *Bifidobacteriaceae* family, *Bifidobacterium longum*, and *Coprococcus eutactus* were decreased in celiac disease patients, while *Bacteroides* genus were more abundant in CD. However, most CD patients were classified as non-dysbiotic in this study [89]. *Coprococcus eutactus* belongs to the *Lachnospiraceae* family. Although *Lachnospiraceae* is among the main producers of short-chain fatty acids, multiple different taxa of *Lachnospiraceae* are also associated with multiple extra- and intra-intestinal diseases, including inflammatory bowel disease [90].

Bodkhe *et al.* found a difference between celiac disease patients and healthy controls at the level of amplicon sequence variants in genera *Helicobacter*, *Prevotella*, *Catenibacterium*, and *Megasphaera*, which were highly abundant, and a decrease of *Barnesiella* genus when compared to HC [91].

Microbiota analysis of adult Mexican celiac disease patient revealed significantly lower abundance of *Bacteroidetes* and *Fusobacteria* in duodenal biopsies of CD patients. Additionally, changes of microbiota after adherence to a gluten-free diet were observed. This included increase of *Pseudomonas* group and other *Proteobacteria* (*Stenophomonas*, *Novosphingobium*) in duodenal samples after diet [92].

A study by Pellegrini *et al.* using a pyrosequencing method of sequencing genetic material showed an increase in the *Firmicutes/Bacteroidetes* ratio and reduction of the *Bacteroidetes* phylum, *Lachnospiraceae*, and *Peptosreptococcaceae* families in CD paediatric patients compared to healthy controls [93]. In contrast, a study by Nistal *et al.* concerning the adult population of celiac disease patients did not show statistically significant difference in the composition of duodenal bacterial communities between CD groups and healthy controls, as each bacterial community was specific to each individual [94].

### NGS – shotgun metagenomic sequencing

A study concerning the microbiota profile of celiac children in Saudi Arabia found an increase of *E. coli* and a decrease of *Desulfovibrio* in faecal samples of celiac disease patients compared to healthy controls. In species analysis, several *Bacteroides* species were significantly decreased in celiac disease patients. Additionally, the study showed an increase of *Micrococcales* order, *Bifidobacterium angulatum* species, and a decrease in *Flavobacteriales* order, *Clostridium* genus, and *Roseburia intestinalis* species in CD group mucosal samples, while faecal samples were characterised by a decrease of *Cardiobacteriales* and *Methanobacteriales* order, *Leuconostocaceae*, *Planctomycetaceae* families, *Tanarella*, and *Citrobacter* genera, and an increase of *Planctomycetaceae* family and *Kocuria* genus [95].

Senicar *et al.*, identifying microbiota population in faecal samples using metagenomic sequencing of previously cultivated samples, showed a decrease of abundance of *Faecalibacterium*, *Bacteroides*, *Roseburia*, *Fusicatenibacter*, *Lachnospiraceae*, *Ruminococcus*, *Butyricoccus*, *Eggerthella*, and *Gordonibacter*, and an increase of abundance of *Blautia*, *Romboutsia*, and *Ruminococcaceae* in CD patients compared to healthy controls [96].

A study of microbiome in infants who later developed celiac disease, while not detecting significant

difference in richness or microorganisms' composition at onset between a celiac disease group and a healthy group, found microorganisms at strain levels that showed different abundances between those groups. Additionally, there were differences in analysis of longitudinal changes in the groups, which shown increased abundance of *Parabacteroides* species and *Lachnospiraceae bacterium* in the celiac disease group [97].

Analysis of microbiota in the adult population of celiac disease patients has shown significant reduction of *Actinobacteria* and *Verrucomicrobia* in treated celiac disease patients. An increase of *Bacteroidetes* abundance was noted in tCD patients with negative transglutaminase serology compared to controls. Celiac disease patients with positive transglutaminase serology had a reduction of *Euryarchaeota* and an increase of *Fusobacteria* abundance compared to controls. There were also differences at species level with Tg- celiac patients having reduced abundance of *Bifidobacterium longum*, *Roseburia* sp. CAG 309, *Ruminococcus bicirculans*, *Ruminococcus ronian*, and *Eubacterium* sp. CAG 274 and increased abundance of *Roseburia inulinivorans*. Tg+ celiac patients had increased abundance of *Veillonella atypica*, *Veillonella tobetsuensis*, *Streptococcus sanguinis*, and *Haemophilus parainfluenzae* and decreased abundance of *Firmicutes bacterium* CAG 83 and *Ruminococcus bicirculans* [98].

### qPCR – quantitative PCR

Collado *et al.* analysed the composition of the *Bifidobacterium* group in active and non-active celiac disease by using qPCR with species-specific probes. This study revealed that total *Bifidobacterium* and *B. longum* numbers were significantly decreased in celiac patients, in both duodenal and stool samples. In addition, *B. breve* and *B. bifidum* were reduced in treated celiac disease patients compared to the active disease group and healthy controls [99]. In another work, analysing multiple bacterial groups in untreated and treated paediatric celiac disease, study of faecal samples revealed that total bacterial counts were significantly lower in uCD and tCD group compared to HC. The duodenal samples similarly had lower total bacterial counts of uCD and tCD, although the difference was not significant. Significantly higher abundance of *Bacteroides* and *Clostridium leptum* groups were noted in uCD and tCD than in the HC group in both faecal and duodenal samples. Additionally, in the untreated group, compared to HC, significantly higher numbers of *E. coli* in faecal and duodenal samples and significantly higher numbers of *Staphylococcus* group in duodenal samples were found. *Bifidobacterium* counts were significantly higher in healthy children compared to untreated celiac patients



[100]. Contrary to other results, a study by Kalliomäki *et al.* of the Finnish paediatric population failed to show differences in the total bacterial count and in the count of *Bacteroides* and *Bifidobacterium* between celiac disease patients and healthy controls [101].

A study analysing the microbiota of adult Iranian celiac patients using faecal samples found that *Bifidobacterium* spp. was the dominant genus, with lower relative abundance in celiac disease patients than in the healthy group. Additionally, patients in the celiac disease group had significantly lower abundance of *Lactobacillus* spp., while having increased abundance of *Firmicutes* phylum [102]. Another study of Iranian celiac patients similarly noted decreased abundance of *Bifidobacterium*, *Firmicutes*, and *Lactobacillus* in celiac disease patients [103].

### FISH/flow cytometry

Because FISH/flow cytometry requires specifically designed probes, studies using this method of microbial detection focus on the main groups of bacteria, frequently described as important for human gut microbiota. Common to these studies are probes specific to *Bifidobacterium* genus, *Lactobacillus/Enterococcus*, *Bacteroides–Prevotella* group, *Streptococcus/Lactococcus*, *Escherichia coli*, *Clostridium histolyticum*, and *Clostridium lituseburense* groups and sulphate-reducing bacteria.

A study by De Palma *et al.*, regarding intestinal dysbiosis in paediatric celiac patients, found that total gram-positive bacterial populations were lowest in untreated celiac disease children and highest in healthy controls, while reaching intermediate values in children who were on a gluten-free diet. Total Gram-negative bacteria reached similar values (ranging from 27.5 to 32.7%) in faeces from the 3 population groups. The ratio of Gram-positive to Gram-negative bacteria was significantly reduced in celiac disease patients, both treated and untreated, compared to healthy controls. *Bacteroides–Prevotella* group and *Bifidobacterium* group proportions were significantly more abundant in untreated CD patients than in healthy controls. The relative abundance of bacteria belonging to *C. histolyticum*, *C. lituseburense*, and *F. prausnitzii* groups were also significantly lower in untreated CD patients than in healthy subjects [104]. This finding is similar to results in microbiota studies of Crohn's disease, where reduction in the *F. prausnitzii* group was observed. *F. prausnitzii* has been shown to produce MAM protein, which has been linked to anti-inflammatory effects, inhibiting activation the NF-κB pathway in intestinal epithelial cells lines [105].

Similar results to previous study were observed in the work of Nadal *et al.*, where the *Bacteroides–Prevotella* group were more abundant in celiac patients

compared to HC. Additionally, the study found that *E. coli* populations were also increased in celiac disease patients. The ratios of beneficial bacterial groups (*Lactobacillus* plus *Bifidobacterium*) to potentially harmful gram-negative bacteria (*Bacteroides–Prevotella* plus *E. coli* groups) detected in biopsy samples were highest in healthy children and lowest in CD patients with active disease, while achieving intermediate values in treated CD [106]. Interestingly, the study (De Palma *et al.*) regarding infants with first-degree relatives with celiac disease also found that there were significantly higher proportions of *Bacteroides–Prevotella* group in children in the high-risk group of developing celiac disease compared to low-risk group. A similar trend was also observed for *E. coli*, *C. lituseburense*, *C. histolyticum*, *Streptococcus–Lactococcus*, *E. rectale–C. coccoides*, and sulphate-reducing bacteria groups [107].

### DGGE/TGGE

Sanz *et al.* found that the diversity of microbiota, according to number of bands in DGGE profiles, was higher in celiac children compared to healthy controls. The DGGE analysis with *Lactobacillus* group-specific primers revealed a higher prevalence of *L. casei* in faecal samples of healthy controls and a higher prevalence of *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, and *Leuconostoc carnosum* in celiac patients. Furthermore, DGGE analysis with *Bifidobacterium* species-specific primers showed that the diversity of *Bifidobacterium* species was significantly higher in healthy patients compared to the celiac group [108]. Similarly, increased diversity of *Bacteroides* was noted in a study by Sánchez *et al.* Additionally, the study showed significantly higher abundance of multiple *Bacteroides* species in HC, including *Bacteroides fragilis* and *Bacteroides uniformis* [109]. The higher diversity of microbiota in celiac children compared to HC was also noted in the analysis of duodenal samples by TGGE method (Schipa *et al.*). The study found increased prevalence of *B. vulgatus* and *E. coli* in CD patients compared to HC [110]. Similarly, Nistal *et al.*, in a study exploring adult celiac populations, showed significantly higher diversity of *Lactobacillus* and *Bifidobacterium* groups in untreated CD patients and healthy controls compared to treated CD patients. A higher presence of *B. bifidum* in the untreated CD group compared to HC and a higher presence of *Bifidobacterium* spp. in HC compared to treated CD patients were also noted [111]. Caminero *et al.*, by means of DGGE analysis of previously cultured bacterial communities from faecal samples, observed a reduction of the *Lactobacillus* group in CD patients compared to HC. Additionally, the *Clostridium* group was significantly higher in the CD group [112].

Di Cagno *et al.* found that PCR-DGGE profiles of *Lactobacillus* and *Bifidobacterium* differed significantly between faecal samples of treated CD and HC children. However, in this study no PCR amplicons were identified by using a primer targeting the *Bifidobacterium* group in samples from duodenal biopsies of both tCD and HC. In contrast, all faecal samples contained *Bifidobacterium* DNA. Further culture-dependent methods showed higher counts of presumptive *Bacteroides*, *Porphyromonas* and *Prevotella*, presumptive staphylococci/micrococci, and *Enterobacteria* in faecal samples of tCD patients [113]. Additionally, the composition and diversity of microbiota may differ according to different symptoms presented by patients. Patients with dermatitis herpetiformis showed different microbiota clustering compared to closer clustering in patients with gastrointestinal symptoms and anaemia [114]. Kopečný *et al.* identified bacteria genera *Streptococcus*, *Legionella*, *Vibrio*, *Streptococcus pneumoniae*, *Clostridium sticklandii*, and *B. uniformis* in faecal samples of CD [115].

### Culturomics

Sánchez *et al.* focused on differences in intestinal *Staphylococcus* spp. between celiac disease children and healthy controls, using a culture-based method and DNA sequencing. *S. epidermidis* were more common in celiac disease patients (both uCD and GFD) than in controls. *Staphylococcus haemolyticus* were more abundant in subjects with active disease than HC. *S. aureus* were less frequent in the uCD group than in other groups, while *Staphylococcus warneri* were less common in GFD-CD. Additionally, *E. faecium* was more abundant in the control group than in both celiac groups [116]. Similarly, a study exploring lactobacilli in celiac patients with use of culture-based and biochemical methods found that faeces of GFD celiac patients showed significantly reduced counts of lactobacilli group compared to healthy children [117]. Sánchez *et al.* noted an increase of phylum *Proteobacteria*, families *Enterobacteriaceae*, *Staphylococcaceae*, and species *S. epidermidis*, *S. pasteurii* in children with active CD compared to both non-active and healthy groups. Additionally, *S. anginosus* and *Streptococcus mutans* groups were more abundant in healthy control group than in the active and non-active celiac groups [118]. Another study, comparing faecal microbiota between celiac and healthy children, observed an increase of *Bacteroides*, *Clostridium*, and *Staphylococcus* genera in the celiac group [119].

### Limitations

In this review we included multiple studies, which in some cases differed in the exact methodology used

in identifying microbiota, which may be a reason for some discrepancies between the results of studies using the same method. Moreover, so far there are no established research standards for the microbiome and the type of samples (faeces or gastrointestinal mucosa – colon or duodenum), so researchers may obtain less consistent results. The age of the studied populations should also be taken into account because it affects the composition of the microbiome.

### Conclusions

In most cases, studies regarding gut microbiota in celiac disease seem to have mostly divergent results, even when comparing studies using the same method of bacteria identification. These disparities may be caused by differences in preparation of samples, differences in studied populations, and differences in status of celiac disease (active vs. inactive). When comparing different methods, the characteristics and drawbacks of each method may influence the results. For example, the results of bacterial identification using culture methods or molecular methods that require previous cultivation of unknown species may suffer from improper transportation of samples or exposure of anaerobic bacteria to an aerobic environment during preparation of samples. Additionally, despite application of culturomics and numerous improvements in culturing methods, we are not yet able to cultivate many of the bacterial species. On the other hand, in the process of targeted amplicon sequencing using the NGS platform, amplification of 16S rRNA requires selection of PCR primers. While universal PCR primers are designed to amplify as many different 16S rRNA gene sequences from as many prokaryotic species as possible, there are no 100% conserved regions of the gene. This may lead to inefficient primer binding and inaccurate sequence detection, which affect the results.

The development of methods for identifying microbiota in disease in recent years presents a chance for a better understanding of the connection between gut bacteria and the pathogenesis of celiac disease. It seems that especially next generation sequencing methods are useful for this task. While shotgun sequencing is generally more accurate than 16S amplicon sequencing, it also is burdened with higher costs. Thus, thanks to lesser costs while maintaining rather high accuracy, NGS 16S amplicon sequencing seems currently to be most useful method of exploring the composition of gut microbiota, despite limitations associated with the method. It is currently recommended as the “gold standard” in research. In our opinion, the least useful method in microbiome research is the culture method, because it has low sensitivity and allows the detection

of only a small part of the bacterial species present in the gastrointestinal tract.

Despite many different results, there are some findings that are mostly consistent across various present-ed studies. Numerous studies confirm that *Lactobacillus* and *Bifidobacterium* genera are less abundant in gut microbiota in celiac disease patients compared to healthy controls. Similarly, increase of abundance of genera *Prevotella*, *Clostridium* and species *E. coli* were consistently noticed in celiac disease patients in multiple studies. In contrast, for *Bacteroidetes* phyla and *Bacteroides* genus, results were inconsistent when comparing various studies. In general, studies using next generation sequencing found multiple genera and species that were not previously linked to celiac disease in works using older methods of bacterial identification. Genera *Alistipes* and *Neisseria* were found to be more abundant in celiac disease in numerous studies using NGS.

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## Ethical approval

Not applicable.

## Conflict of interest

The authors declare no conflict of interest.

## References

- Thursby E, Juge N. Introduction to the human gut microbiota. *Biochem J* 2017; 474: 1823-36.
- Ley RE, Peterson DA, Gordon JL. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 2006; 124: 837-48.
- Li J, Jia H, Cai X, et al. An integrated catalog of reference genes in the human gut microbiome. *Nature Biotechnol* 2014; 32: 834-41.
- Almeida A, Nayfach S, Boland M, et al. A unified catalog of 204,938 reference genomes from the human gut microbiome. *Nat Biotechnol* 2021; 39: 105-14.
- Hugon P, Dufour JC, Colson P, et al. A comprehensive repertoire of prokaryotic species identified in human beings. *Lancet Infect Dis* 2015; 15: 1211-9.
- Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* 2010; 10: 159-69.
- Salzman NH. Microbiota-immune system interaction: an uneasy alliance. *Curr Opin Microbiol* 2011; 14: 99-105.
- Odamaki T, Kato K, Sugahara H, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol* 2016; 16: 90.
- Lin D, Wang R, Luo J, et al. The core and distinction of the gut microbiota in Chinese populations across geography and ethnicity. *Microorganisms* 2020; 8: 1579.
- Savin Z, Kivity S, Yonath H, Yehuda S. Smoking and the intestinal microbiome. *Arch Microbiol* 2018; 200: 677-84.
- Graf D, Di Cagno R, Fåk F, et al. Contribution of diet to the composition of the human gut microbiota. *Microb Ecol Health Dis* 2015; 26: 26164.
- Wilson AS, Koller KR, Ramaboli MC, et al. Diet and the human gut microbiome: an international review. *Dig Dis Sci* 2020; 65: 723-40.
- Gomaa EZ. Human gut microbiota/microbiome in health and diseases: a review. *Antonie van Leeuwenhoek* 2020; 113: 2019-40.
- Tooley KL. Effects of the human gut microbiota on cognitive performance, brain structure and function: a narrative review. *Nutrients* 2020; 12: 3009.
- Shen J, Obin MS, Zhao L. The gut microbiota, obesity and insulin resistance. *Mol Aspects Med* 2013; 34: 39-58.
- Distruiti E, Monaldi L, Ricci P, Fiorucci S. Gut microbiota role in irritable bowel syndrome: new therapeutic strategies. *World J Gastroenterol* 2016; 22: 2219-41.
- Bastiaanssen TFS, Cowan CSM, Claesson MJ, et al. Making sense of ... the microbiome in psychiatry. *Int J Neuropsychopharmacol* 2019; 22: 37-52.
- Green PHR, Cellier C. Celiac disease. *N Engl J Med* 2007; 357: 1731-43.
- Lionetti E, Catassi C. The role of environmental factors in the development of celiac disease: what is new? *Diseases* 2015; 3: 282-93.
- Parzanese I, Qehajaj D, Patrinicola F, et al. Celiac disease: from pathophysiology to treatment. *World J Gastrointest Pathophysiol* 2017; 8: 27-38.
- Caminero A, Herrán AR, Nistal E, et al. Diversity of the cultivable human gut microbiome involved in gluten metabolism: isolation of microorganisms with potential interest for coeliac disease. *FEMS Microbiol Ecol* 2014; 88: 309-19.
- Chelakkot C, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. *Exp Mol Med* 2018; 50: 1-9.
- Mozafarybazargany M, Khonsari M, Sokoty L, et al. The effects of probiotics on gastrointestinal symptoms and microbiota in patients with celiac disease: a systematic review and meta-analysis on clinical trials. *Clin Exp Med* 2023; 23: 2773-88.
- Segura V, Ruiz-Carnicer Á, Sousa C, et al. New insights into non-dietary treatment in celiac disease: emerging therapeutic options. *Nutrients* 2021; 13: 2146.
- Tang Q, Jin G, Wang G, et al. Current sampling methods for gut microbiota: a call for more precise devices. *Front Cell Infect Microbiol* 2020; 10: 151.
- Margulies M, Egholm M, Altman WE, et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005; 437: 376-80.
- Deurenberg RH, Bathoorn E, Chlebowicz MA, et al. Application of next generation sequencing in clinical microbiology and infection prevention. *J Biotechnol* 2017; 243: 16-24.

28. Strom SP. Current practices and guidelines for clinical next-generation sequencing oncology testing. *Cancer Biol Med* 2016; 13: 3-11.
29. Zhang W, Cui H, Wong LJC. Application of next generation sequencing to molecular diagnosis of inherited diseases. *Top Curr Chem* 2014; 336: 19-45.
30. van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation sequencing technology. *Trends Genetics* 2014; 30: 418-26.
31. Kanzi AM, San JE, Chimukangara B, et al. Next generation sequencing and bioinformatics analysis of family genetic inheritance. *Front Genet* 2020; 11: 544162.
32. Cao Y, Fanning S, Proos S, et al. A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. *Front Microbiol* 2017; 8: 1829.
33. Grada A, Weinbrecht K. Next-generation sequencing: methodology and application. *J Invest Dermatol* 2013; 133: e11.
34. Head SR, Komori HK, LaMere SA, et al. Library construction for next-generation sequencing: overviews and challenges. *BioTechniques* 2014; 56: 61-77.
35. Knierim E, Lucke B, Schwarz JM, et al. Systematic comparison of three methods for fragmentation of long-range PCR products for next generation sequencing. *PLoS One* 2011; 6: e28240.
36. Hu T, Chitnis N, Monos D, Dinh A. Next-generation sequencing technologies: an overview. *Human Immunol* 2021; 82: 801-11.
37. Slatko BE, Gardner AF, Ausubel FM. Overview of next generation sequencing technologies. *Curr Protoc Mol Biol* 2018; 122: e59.
38. Magi A, Benelli M, Gozzini A, et al. Bioinformatics for next generation sequencing data. *Genes* 2010; 1: 294-307.
39. Petrosino JF, Highlander S, Luna RA, et al. Metagenomic pyrosequencing and microbial identification. *Clin Chem* 2009; 55: 856-66.
40. Drancourt M, Raoult D. Sequence-based identification of new bacteria: a proposition for creation of an orphan bacterium repository. *J Clin Microbiol* 2005; 43: 4311-5.
41. Sanschagrin S, Yergeau E. Next-generation sequencing of 16S ribosomal RNA gene amplicons. *J Vis Exp* 2014; 90: 51709.
42. Gupta S, Mortensen MS, Schjørring S, et al. Amplicon sequencing provides more accurate microbiome information in healthy children compared to culturing. *Commun Biol* 2019; 2: 291.
43. Boers SA, Jansen R, Hays JP. Understanding and overcoming the pitfalls and biases of next-generation sequencing (NGS) methods for use in the routine clinical microbiological diagnostic laboratory. *Eur J Clin Microbiol Infect Dis* 2019; 38: 1059-70.
44. Ranjan R, Rani A, Metwally A, et al. Analysis of the microbiome: advantages of whole genome shotgun versus 16S amplicon sequencing. *Biochem Biophys Res Commun* 2016; 469: 967-77.
45. Weinroth MD, Belk AD, Dean C, et al. Considerations and best practices in animal science 16S ribosomal RNA gene sequencing microbiome studies. *J Animal Sci* 2022; 100: skab346.
46. Sharpton TJ. An introduction to the analysis of shotgun metagenomic data. *Front Plant Sci* 2014; 5: 209.
47. Wensel CR, Pluznick JL, Salzberg SL, Sears CL. Next-generation sequencing: insights to advance clinical investigations of the microbiome. *J Clin Invest* 2022; 132: e154944.
48. Navarro E, Serrano-Heras G, Castaño MJ, Solera J. Real-time PCR detection chemistry. *Clin Chim Acta* 2015; 439: 231-50.
49. Kralik P, Ricchi M. A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. *Front Microbiol* 2017; 8: 108.
50. Bonk F, Popp D, Harms H, Centler F. PCR-based quantification of taxa-specific abundances in microbial communities: quantifying and avoiding common pitfalls. *J Microbiol Methods* 2018; 153: 139-47.
51. Rezasoltani S, Ahmadi Bashirzadeh D, Nazemalhosseini Mojarad E, et al. Signature of gut microbiome by conventional and advanced analysis techniques: advantages and disadvantages. *Middle East J Dig Dis* 2020; 12: 5-11.
52. Moter A, Göbel UB. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J Microbiol Methods* 2000; 41: 85-112.
53. La Reau AJ, Strom NB, Filvaroff E, et al. Shallow shotgun sequencing reduces technical variation in microbiome analysis. *Sci Rep* 2023; 13: 7668.
54. Hillmann B, Al-Ghalith GA, Shields-Cutler RR, et al. Evaluating the information content of shallow shotgun metagenomics. *Msystems* 2018; 3: e00069-18.
55. Frickmann H, Zautner AE, Moter A, et al. Fluorescence in situ hybridization (FISH) in the microbiological diagnostic routine laboratory: a review. *Crit Rev Microbiol* 2017; 43: 263-93.
56. Arrigucci R, Bushkin Y, Radford F, et al. FISH-Flow, a protocol for the concurrent detection of mRNA and protein in single cells using fluorescence in situ hybridization and flow cytometry. *Nat Protoc* 2017; 12: 1245-60.
57. Yilmaz S, Haroon MF, Rabkin BA, et al. Fixation-free fluorescence in situ hybridization for targeted enrichment of microbial populations. *ISME J* 2010; 4: 1352-6.
58. Muyzer G, Smalla K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* 1998; 73: 127-41.
59. Green SJ, Leigh MB, Neufeld JD. Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. In: *Handbook of Hydrocarbon and Lipid Microbiology*. Timmis KN (ed.). Springer 2010; 4137-58.
60. Nakatsu CH. Soil microbial community analysis using denaturing gradient gel electrophoresis. *Soil Sci Soc Am J* 2007; 71: 562-71.
61. Lagier JC, Dubourg G, Million M, et al. Culturing the human microbiota and culturomics. *Nat Rev Microbiol* 2018; 16: 540-50.
62. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the human intestinal microbial flora. *Science* 2005; 308: 1635-8.
63. Browne HP, Forster SC, Anonye BO, et al. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature* 2016; 533: 543-6.
64. Lagier JC, Khelaifia S, Alou MT, et al. Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat Microbiol* 2016; 1: 16203.

65. Diakite A, Dubourg G, Dione N, et al. Optimization and standardization of the culturomics technique for human microbiome exploration. *Sci Rep* 2020; 10: 9674.
66. Dione N, Khelaifia S, La Scola B, et al. A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology. *Clin Microbiol Infect* 2016; 22: 53-8.
67. Seng P, Drancourt M, Gouriet F, et al. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis* 2009; 49: 543-51.
68. Singh P, Rawat A, Al-Jarrah B, et al. Distinctive microbial signatures and gut-brain crosstalk in pediatric patients with celiac disease and type 1 diabetes mellitus. *Int J Mol Sci* 2021; 22: 1511.
69. Quagliarello A, Aloisio I, Bozzi Cionci N, et al. Effect of Bifidobacterium breve on the intestinal microbiota of celiac children on a gluten free diet: a pilot study. *Nutrients* 2016; 8: 660.
70. Whittaker RH. Evolution and measurement of species diversity. *Taxon* 1972; 21: 213-51.
71. Rastogi S, Singh A. Gut microbiome and human health: exploring how the probiotic genus *Lactobacillus* modulate immune responses. *Front Pharmacol* 2022; 13: 1042189.
72. Iljazovic A, Roy U, Gálvez EJC, et al. Perturbation of the gut microbiome by *Prevotella* spp. enhances host susceptibility to mucosal inflammation. *Mucosal Immunol* 2021; 14: 113-24.
73. Henke MT, Kenny DJ, Cassilly CD, et al. *Ruminococcus gnavus*, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. *Proc Natl Acad Sci USA* 2019; 116: 12672-7.
74. Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Lett* 2014; 588: 4223-33.
75. Zafeiropoulou K, Nichols B, Mackinder M, et al. Alterations in intestinal microbiota of children with celiac disease at the time of diagnosis and on a gluten-free diet. *Gastroenterology* 2020; 159: 2039-51.e20.
76. Primec M, Klemenak M, Di Gioia D, et al. Clinical intervention using Bifidobacterium strains in celiac disease children reveals novel microbial modulators of TNF- $\alpha$  and short-chain fatty acids. *Clin Nutr* 2019; 38: 1373-81.
77. Sample D, Foughse J, King S, et al. Baseline fecal microbiota in pediatric patients with celiac disease is similar to controls but dissimilar after 1 year on the gluten-free diet. *JPGN Rep* 2021; 2: e127.
78. Girdhar K, Dogru YD, Huang Q, et al. Dynamics of the gut microbiome, IgA response, and plasma metabolome in the development of pediatric celiac disease. *Microbiome* 2023; 11: 9.
79. Millettich PL, Ahrens AP, Russell JT, et al. Gut microbiome markers in subgroups of HLA class II genotyped infants signal future celiac disease in the general population: ABIS study. *Front Cell Infect Microbiol* 2022; 12: 920735.
80. Olivares M, Walker AW, Capilla A, et al. Gut microbiota trajectory in early life may predict development of celiac disease. *Microbiome* 2018; 6: 36.
81. Rintala A, Riikonen I, Toivonen A, et al. Early fecal microbiota composition in children who later develop celiac disease and associated autoimmunity. *Scand J Gastroenterol* 2018; 53: 403-9.
82. Constante M, Libertucci J, Galipeau HJ, et al. Biogeographic variation and functional pathways of the gut microbiota in celiac disease. *Gastroenterology* 2022; 163: 1351-63.e15.
83. Mirsepasi-Lauridsen HC, Vallance BA, Krogfelt KA, Petersen AM. *Escherichia coli* pathobionts associated with inflammatory bowel disease. *Clin Microbiol Rev* 2019; 32: 10.1128/cmr.00060-18.
84. Panelli S, Capelli E, Lupo GFD, et al. Comparative study of salivary, duodenal, and fecal microbiota composition across adult celiac disease. *J Clin Med* 2020; 9: 1109.
85. Nobel YR, Rozenberg F, Park H, et al. Lack of effect of gluten challenge on fecal microbiome in patients with celiac disease and non-celiac gluten sensitivity. *Clin Transl Gastroenterol* 2021; 12: e00441.
86. Nylund L, Hakkola S, Lahti L, et al. Diet, perceived intestinal well-being and compositions of fecal microbiota and short chain fatty acids in oat-using subjects with celiac disease or gluten sensitivity. *Nutrients* 2020; 12: 2570.
87. D'Argenio V, Casaburi G, Precone V, et al. Metagenomics reveals dysbiosis and a potentially pathogenic *N. flavescens* strain in duodenum of adult celiac patients. *Am J Gastroenterol* 2016; 111: 879-90.
88. Shi T, Feng Y, Liu W, et al. Characteristics of gut microbiota and fecal metabolomes in patients with celiac disease in Northwest China. *Front Microbiol* 2022; 13: 1020977.
89. Palmieri O, Castellana S, Bevilacqua A, et al. Adherence to gluten-free diet restores alpha diversity in celiac people but the microbiome composition is different to healthy people. *Nutrients* 2022; 14: 2452.
90. Vacca M, Celano G, Calabrese FM, et al. The controversial role of human gut Lachnospiraceae. *Microorganisms* 2020; 8: 573.
91. Bodkhe R, Shetty SA, Dhotre DP, et al. Comparison of small gut and whole gut microbiota of first-degree relatives with adult celiac disease patients and controls. *Front Microbiol* 2019; 10: 164.
92. Garcia-Mazcorro JF, Rivera-Gutierrez X, Cobos-Quevedo ODJ, et al. First insights into the gut microbiota of Mexican patients with celiac disease and non-celiac gluten sensitivity. *Nutrients* 2018; 10: 1641.
93. Pellegrini S, Sordi V, Bolla AM, et al. Duodenal mucosa of patients with type 1 diabetes shows distinctive inflammatory profile and microbiota. *J Clin Endocrinol Metab* 2017; 102: 1468-77.
94. Nistal E, Caminero A, Herrán AR, et al. Study of duodenal bacterial communities by 16S rRNA gene analysis in adults with active celiac disease vs non-celiac disease controls. *J Appl Microbiol* 2016; 120: 1691-700.
95. El Mouzan M, Al-Hussaini A, Serena G, et al. Microbiota profile of new-onset celiac disease in children in Saudi Arabia. *Gut Pathogens* 2022; 14: 37.
96. Senicar T, Kukovic A, Tkalec V, et al. Comparison of microbial populations in saliva and feces from healthy and celiac adolescents with conventional and molecular approaches after

- cultivation on gluten-containing media: an exploratory study. *Microorganisms* 2021; 9: 2375.
97. Leonard MM, Valitutti F, Karathia H, et al. Microbiome signatures of progression toward celiac disease onset in at-risk children in a longitudinal prospective cohort study. *Proc Natl Acad Sci USA* 2021; 118: e2020322118.
  98. Francavilla A, Ferrero G, Pardini B, et al. Gluten-free diet affects fecal small non-coding RNA profiles and microbiome composition in celiac disease supporting a host-gut microbiota crosstalk. *Gut Microbes* 2023; 15: 2172955.
  99. Collado MC, Donat E, Ribes-Koninckx C, et al. Imbalances in faecal and duodenal *Bifidobacterium* species composition in active and non-active coeliac disease. *BMC Microbiol* 2008; 8: 232.
  100. Collado MC, Donat E, Ribes-Koninckx C, et al. Specific duodenal and faecal bacterial groups associated with paediatric coeliac disease. *J Clin Pathol* 2009; 62: 264-9.
  101. Kalliomäki M, Satokari R, Lähteenoja H, et al. Expression of microbiota, toll-like receptors, and their regulators in the small intestinal mucosa in celiac disease. *J Pediatr Gastroenterol Nutr* 2012; 54: 727-32.
  102. Naseri K, Dabiri H, Olfatfar M, et al. Evaluation of gut microbiota of Iranian patients with celiac disease, non-celiac wheat sensitivity, and irritable bowel syndrome: are there any similarities? *BMC Gastroenterol* 2023; 23: 15.
  103. Soheilian-Khorzoghi M, Rezasoltani S, Moheb-Alian A, et al. Impact of nutritional profile on gut microbiota diversity in patients with celiac disease. *Curr Microbiol* 2022; 79: 129.
  104. De Palma G, Nadal I, Medina M, et al. Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. *BMC Microbiol* 2010; 10: 63.
  105. Quévrain E, Maubert MA, Michon C, et al. Identification of an anti-inflammatory protein from *Faecalibacterium prausnitzii*, a commensal bacterium deficient in Crohn's disease. *Gut* 2016; 65: 415-25.
  106. Nadal I, Donat E, Ribes-Koninckx C, et al. Imbalance in the composition of the duodenal microbiota of children with coeliac disease. *J Med Microbiol* 2007; 56: 1669-74.
  107. De Palma G, Capilla A, Nadal I, et al. Interplay between human leukocyte antigen genes and the microbial colonization process of the newborn intestine. *Curr Issues Mol Biol* 2010; 12: 1-10.
  108. Sanz Y, Sánchez E, Marzotto M, et al. Differences in faecal bacterial communities in coeliac and healthy children as detected by PCR and denaturing gradient gel electrophoresis. *FEMS Immunol Med Microbiol* 2007; 51: 562-8.
  109. Sanchez E, Donat E, Ribes-Koninckx C, et al. Intestinal *Bacteroides* species associated with coeliac disease. *J Clin Pathol* 2010; 63: 1105-11.
  110. Schippa S, Iebba V, Barbato M, et al. A distinctive "microbial signature" in celiac pediatric patients. *BMC Microbiol* 2010; 10: 175.
  111. Nistal E, Caminero A, Vivas S, et al. Differences in faecal bacteria populations and faecal bacteria metabolism in healthy adults and celiac disease patients. *Biochimie* 2012; 94: 1724-9.
  112. Caminero A, Nistal E, Herrán AR, et al. Differences in gluten metabolism among healthy volunteers, coeliac disease patients and first-degree relatives. *Br J Nutr* 2015; 114: 1157-67.
  113. Di Cagno R, De Angelis M, De Pasquale I, et al. Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization. *BMC Microbiol* 2011; 11: 219.
  114. Wacklin P, Kaukinen K, Tuovinen E, et al. The duodenal microbiota composition of adult celiac disease patients is associated with the clinical manifestation of the disease. *Inflamm Bowel Dis* 2013; 19: 934-41.
  115. Kopečný J, Mrázek J, Fliegerová K, et al. The intestinal microflora of childhood patients with indicated celiac disease. *Folia Microbiol* 2008; 53: 214-6.
  116. Sánchez E, Ribes-Koninckx C, Calabuig M, Sanz Y. Intestinal *Staphylococcus* spp. and virulent features associated with coeliac disease. *J Clin Pathol* 2012; 65: 830-4.
  117. Lorenzo Pisarello MJ, Vintiñi EO, González SN, et al. Decrease in lactobacilli in the intestinal microbiota of celiac children with a gluten-free diet, and selection of potentially probiotic strains. *Can J Microbiol* 2015; 61: 32-7.
  118. Sánchez E, Donat E, Ribes-Koninckx C, et al. Duodenal-mucosal bacteria associated with celiac disease in children. *Appl Environ Microbiol* 2013; 79: 5472-9.
  119. Caminero A, Herrán AR, Nistal E, et al. Diversity of the cultivable human gut microbiome involved in gluten metabolism: isolation of microorganisms with potential interest for coeliac disease. *FEMS Microbiol Ecol* 2014; 88: 309-19.

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