Comparison of Small Gut and Whole Gut Microbiota of First-Degree Relatives with Adult Celiac Disease Patients and Controls

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Abstract

Recent studies on celiac disease (CeD) have shown the role of gut microbiota alterations in CeD pathogenesis. Whether this alteration in the microbial community is the cause or effect of the disease is not well understood, especially in adult onset of disease. The first-degree relatives (FDRs) of CeD patients may provide an opportunity to study gut microbiome in pre-disease state as FDRs are genetically susceptible to CeD. By using 16S rRNA gene sequencing, we observed that ecosystem level diversity measures (except in the duodenum) were not significantly different between the disease condition (CeD), pre-disease (FDR) and control subjects. However, differences were observed at the level of amplicon sequence variant (ASV), suggesting alterations in specific taxa between pre-diseases and diseased condition. Duodenal biopsies showed higher differences in ASVs compared to faecal samples indicating larger disruption of microbiota at disease site. Increased abundance of specific *Helicobacter* ASVs were observed in duodenum of CeD when compared to FDR ($p < 0.01$). In case of fecal samples CeD microbiome is characterized by reduced abundance of beneficial taxa such as *Akkermansia, Ruminococcus* and *Actinomyces*. In addition, predicted functional metagenome showed reduced ability of gluten degradation by CeD faecal microbiota in comparison to FDRs and controls.

Introduction

Celiac disease (CeD) is a common, chronic immune mediated enteropathy of the small intestine which affects approximately 0.7% of the global population (Singh et al., in press). Once thought to be uncommon in Asia, CeD is now prevalent in many Asian countries including India (Makharia et al., 2011). CeD is caused by the consumption of gluten proteins present in cereals such as wheat, barley and rye in genetically susceptible individuals (Caminero et al., 2015). While many genes are involved in the development of CeD, thus far only the presence of HLA-DQ2 or DQ8 haplotype is considered to be essential (Sanz and Pama, 2011). Additional factors that contribute to pathogenesis include other co-genetic factors (genome wide association studies have identified several markers), wheat-related factors (age of ingestion, type and quantity of wheat) and the way gluten is metabolized in the intestine (Kagnoff, 2007; van de Wal et al., 1998; Verdu et al., 2015). About 30-40% of the gluten protein consists of glutamine and proline. Since humans are unable to enzymatically break the molecular bonds between these two amino-acids, many immunogenic peptides are produced (Jabri and Sollid, 2006). There remains a possibility that enzymes secreted by the small intestinal microbiota convert some of these immunogenic peptides to non-immunogenic peptides.

While 20-30% of individuals in many countries including India are genetic susceptibility to develop CeD and the majority of them are exposed to wheat, only 1% of them develop CeD. This brings forth the role of other factors such as the gut microbiota in the pathogenesis of CeD (Sánchez et al., 2012). Recently, numerous studies have highlighted the potential role of gut microbiota in inflammatory gastrointestinal diseases (de Sousa Moraes et al., 2014; Fernandez-Feo et al., 2013; Png et al., 2010; Rivière et al., 2016; Schneeberger et al., 2015; Zeng et al., 2017).

However, these changes in the microbial community structure and function in patients with CeD are cause or effect of the disease state remains unclear to date. In order to answer this question, one has to examine the status of the gut microbiota in the pre-disease state. Recently two studies investigated the microbiota of at risk children who developed CeD few years after birth. One study observed an increase in *Bifidobacterium breve* and *Enterococcus* spp. in infants that developed active CeD (Olivares et al., 2018). Another study, did not observe any association between microbiota composition and development of CeD during the age of 9 and 12 months (Rintala et al., 2018). However, potential microbiota related triggers for development of CeD in later adult life still remain unclear. While 70-80% percent of first-degree relatives (FDRs) of patients with CeD have HLADQ2/DQ8 haplotype (compared to 30% in the general population); only approximately 8.5% of FDRs develop CeD (Singh et al., 2015). Thus, the question arises; why do only few FDRs develop CeD and what is the role of the gut microbiome in disease protection? Indirect evidence of altered microbiota in relatives of patients with CeD is suggested by significantly lower levels of acetic acid and total short chain fatty acids, and higher faecal tryptic activity (Tjellström et al., 2007). Nevertheless, to date there is no information on the gut microbial composition and function in FDRs of patients with CeD, especially using the latest sequencing approaches. Additionally, it is important to explore the status of the microbiota in both the small intestine, the site of the disease, and feces, as representative of whole gut microbiome. To test the hypothesis that gut microbiome of FDR is different from CeD and could potentially play an important role in the pathogenesis of CeD, we explored the composition of both small intestinal and the whole gut microbiome using Illumina MiSeq in a subset of patients with CeD, first degree relatives and controls. We further investigated the potential microbial functions that are characteristic of FDR and CeD microbiota.

Results

Comparison of faecal and duodenal microbial community in the study cohort

The characteristics of the study subjects have been summarized in the Table 1. All the participants were on staple gluten containing diet during sampling for this particular study. After diagnosis of CeD the patients underwent therapy with dietary recommendation to avoid gluten in daily diet. However, in the present study, we do not include samples after dietary changes. Both duodenal biopsies and faecal samples were included to investigate differences in both site-specific and whole gut bacterial diversity and community structure in patients with CeD, FDRs and controls. The microbial community was different between the faecal and duodenal biopsies irrespective of whether they were from CeD, FDR or DC groups (Supplementary figure S1a), (Analysis of similarities; ANOSIM statistic R: 0.4998, Significance: 0.001). Analysis of alpha diversity between the sampling sites suggested no significant differences between the sampling sites (Supplementary figure S1b). Further analyses were carried out separately for faecal and duodenal samples in different groups.

Site specific bacterial community structure in FDRs, CeD and controls

Duodenal and faecal microbiota composition and structure is distinct in FDRs, CeD and control groups:

To investigate if patients with CeD, FDRs or DC had site specific dissimilarities in microbiota composition, we analyzed microbiome composition of duodenal and faecal samples separately. Alpha diversity was determined using Shannon index, pairwise comparisons of alpha diversity in duodenal biopsies between FDRs, CeD and controls suggested no significant differences (Figure 1a). Similarly, for faecal samples no significant differences were observed for alpha diversity between diagnosis groups (Figure 1b).

Further, unconstrained comparison based on Bray-Curtis revealed no significant separation for duodenal biopsy microbiota between CeD, FDRs and control samples (Analysis of similarities; Anosim test; R-statistic =0.0014, p = 0.427 Figure 1c). In case of faecal microbiome, comparison based on Bray-Curtis distances between diagnosis groups was done. Similar to the duodenal biopsy microbiome it was not significantly different between diagnosis groups (Analysis of similarities; Anosim test; R-statistic = 0.051, p = 0.058 Figure 1d).

Taxonomic differences in microbiota from duodenal biopsies of FDRs CeD, and controls:

At phylum level in duodenal biopsy samples Actinobacteria, Bacteroides, Euryarchaeota, Firmicutes and Proteobacteria were the dominant members (Additional file figure S2). When performed pairwise comparison Actinobacteria (p= 0.013) and Bacteroides (0.02) were found be significantly increased in predisease state (FDR) in comparison to controls. Moreover, at order level FDR showed significant more abundance of Actinomycetales and Clostridiales than the control duodenal biopsies (p< 0.05) (Additional file figure S3).

To further investigate differences at lower taxonomic level between diagnosis groups, we used the DESeq2 package with default parameters.

Changes in taxonomic abundance in the biopsies of FDRs in comparison to controls:

Differential abundance analysis identified bacterial genera *Ruminococcus, Intestinibacter, Eubacterium* and *Anaerostipes* belonging to Clostridiales to be at least 21 fold higher in abundance in FDR biopsies (Figure 2a). Order Actinomycetales (p=0.02) and its genus *Actinomyces* were also observed in higher abundance in FDRs in comparison with controls. Notably, we observed differentially higher abundance of opportunistic pathogenic genera

Helicobacter and *Prevotella* in duodenum of FDRs (>23 fold change, $p < 0.01$). In total a group 17 genera were significantly more abundant in FDR biopsy samples in comparison to control biopsies ($p< 0.01$) and these genera were at least 21 fold more in abundance. However, on the other side this analysis also identified 10 genera that were significantly depleted in FDR samples (>log2 Fold Change of 20, $p < 0.01$), including ASVs belonging to *Ruminococcus, Blautia, Eubacterium* and *Intestinibacter*. Among these the most significant difference in a bacterial genera was *Eubacterium* which was 26 fold decreased in FDR samples ($p < 0.01$).

Changes in taxonomic abundance in the biopsies of patient with Celiac disease in comparison to controls:

Next we compared microbial composition between CeD and controls to explore differentially abundant and reduced taxa in disease state. 35 ASVs were found to be at least 22-fold higher in abundance in duodenal biopsies of CeD group than the control biopsy samples. These ASVs were belonging to *Blautia, Catenibacter, Helicobacter, Lactobacillus, Megasphaera, Methanomassillicoccus* and *Prevotella* (Figure 2b). The most significant difference in a bacterial species were associated with *Lactobacillus, Methanomassiliicoccus, Catenibacter* and opportunistic pathogen *Helicobacter,* which were more than 22 fold higher in abundance in CeD biopsy samples than those of control samples ($p < 0.01$). Furthermore, *Megasphaera* and *Blautia* genera were also in higher abundance in CeD samples. Analysis also identified 34 ASVs belonging to 9 genera that were significantly depleted in CeD samples ($p < 0.01$). The majority of these genera (4/9) were belonging to the orders Clostridiales including *genera Ruminococcus, Intestinibacter, Blautia* and *Eubacterium*. Among these, the most depleted taxon was the short chain fatty acid (SCFA) producer *Ruminococcus*, which was 24 fold reduced in samples from

those with CeD ($p< 0.01$). Moreover, higher abundance of genus *Turicibacter,* and *Moraxella* was significantly associated with a control microbial configuration in comparison with CeD.

Changes in taxonomic abundance in the biopsies of patient with Celiac disease in comparison to First degree relatives of CeD:

Next, to identify the differentially abundant taxa between predisease and disease state we did similar analysis for CeD and FDR groups. DESeq2 identified a group of 27 taxa belonging to Firmicutes and Proteobacteria that were significantly more abundant in CeD duodenal samples. These taxa were found to be at least 22-fold higher in abundance and were belonging to genera *Blautia, Eubacterium, Helicobacter, Lactobacillus, Megasphaera* and *Akkermansia*. Similar to the comparison with controls, bacterial genera *Methanomassillicoccus, Catenibacter* and *Helicobacter* were the most significantly abundant bacterial genera in CeD duodenum samples in comparison to FDRs (>24 fold change, $p < 0.01$). In addition, *Moraxella* and *Eubacterium* were also the other most differential abundant taxa were associated with duodenum in disease condition (>24 fold change, $p < 0.01$).

We also identified 59 taxa belonging Firmicutes and Actinobacteria were significantly depleted in CeD samples ($p < 0.01$). Also the order Clostridiales and the beneficial genera affiliated to it such as *Ruminococcus, Intestinibacter* and *Anaerostipes* were also significantly reduced in CeD biopsies.

Moreover, *Gemella* a commensal genus of the upper respiratory tract, gluten degrader *Actinomyces* and genera *Streptococcus* and *Bifidobacterium* were also found to be significantly low in abundance in CeD (Figure 2c).

Taxonomic differences in the faecal microbiota in patients with CeD, FDRs and controls

Phylum level comparison of microbial community between CeD, FDRs and controls demonstrated that Proteobacteria, Actinobacteria, Bacteroidetes, Euryarchaeota and Firmicutes constitute the majority of the faecal microbiota (Additional file figure S4). However in contrast to the biopsy samples Bacteroidetes was found to be marginally decreased in FDR samples in comparison to controls (p= 0.058). Similar trend was observed for order Bacteroidales, it showed marginal lower abundance in FDRs (p= 0.054). However, order Clostridiales was significantly abundant in FDRs in comparison to controls (p= 0.017) (Additional file figure S5).

Changes in taxonomic abundance in the feces of FDR in comparison with Controls

In faeces of FDRs mostly the significant depletion (22/30) of beneficial taxa was observed. Only the archaeal genus *Methanomassiliicoccus* was observed differentially abundant in FDRs faecal samples than those of control samples (Figure 3a). However, 7 ASVs belonging to same genus were significantly reduced in FDRs. Further analysis identified more than 23 fold ($p < 0.01$) reduction in bacterial genera which are known for a healthy microbiota homeostasis, which include *Akkermansia, Lactobacillus* and *Dorea*.

Changes in taxonomic abundance in the feces of CeD in comparison with Controls

Similar to the FDRs, mostly the depletion of bacterial taxa was observed in CeD faecal samples when compared with controls. Moreover, the same ASVs of *Akkermansia, Lactobacillus* and *Dorea* were significantly depleted in CeD (Figure 3b). In addition *Prevotella* showed 23 fold (p< 0.01) reductions in abundance in CeD. On the other hand, DESeq2 identified genus *Lactobacillus* to be in significant abundance in disease condition (CeD) in comparison to control fecal samples.

208 **Changes in taxonomic abundance in the feces of CeD in comparison with FDRs**

209 To explore differentially abundant taxa in disease condition in comparison to predisease state, we
210 compared microbial composition between CeD and FDRs. In disease state mostly a significant
211 depletion was observed for physiologically important bacterial taxa compared with FDRs faeces
212 (Figure 3c). Order Clostridiales and genera *Intestinibacter, Dorea* and *Blautia* belonging to this
213 order were significantly in lower abundance in CeD. In addition, *Pediococcus* was found to be
214 23 fold reduced abundance in CeD, however ASVs affiliated with *Lactobacillus* were more than
215 24 fold differentially abundant in CeD in comparison FDRs.

216

217 **Imputed metagenome of FDR and CeD duodenal microbiome shows reduced proportion of**
218 **genes involved in gluten metabolism in comparison to that of the controls**

219 In addition to differentially abundant microbial taxa, different study groups might have altered
220 metabolic potential. Of specific interest were the enzymes related to glutenases as they play a
221 role in breakdown of gliadin residues. We followed Piphillin workflow to predict functional
222 profile of fecal microbial community (Iwai et al., 2016). A total of 159 KEGG orthologies (KO)
223 were significantly different between diagnosis groups (Supplementary Table 1). Among these the
224 KO abundance for Xaa-pro dipeptidase (K01271, Prolidase) enzyme which is known to have role
225 in gluten degradation was found to be significantly reduced in CeD as compared to FDR and
226 controls (figure 4).

227

228 **Discussion**

229 The aim of the present study was to investigate differences in the duodenal and faecal microbiota
230 of pre-diseased state i.e. FDRs subjects compared to diseased state i.e. CeD and controls. The

231 FDR group was included for two main reasons: 1) They represent a population which is
232 genetically-susceptible to develop CeD; 2) They provide a unique opportunity to identify
233 features of the host as well as of the associated microbiota that may be involved in the protection
234 against developing CeD. We collected both duodenal biopsies and faecal samples to investigate
235 both local and overall changes in the microbiota in FDR, patients with CeD and controls.
236 To the best of our knowledge, reports on site specific microbiota patterns in adult patients with
237 CeD remain scarce, and no results on both site specific and whole gut microbiome on FDRs have
238 been reported to date. Present study provides an overall view on differences of both site-specific
239 changes as well as changes in the faecal microbiota of FDRs, CeD and DC.
240 At lower taxonomic level, several taxa were identified to be differentially abundant between the
241 diagnosis groups. Notably, ASVs related to *Helicobacter, Ruminococcus*, *Megasphaera,* and
242 *Lactobacillus,* showed higher (> 24) log2 fold change in CeD biopsy samples. When we
243 performed analysis at species level, abundance of an ASV1811, *H. pylori* was higher in CeD
244 compared to controls and FDR subjects. In turn FDR were found to harbor higher abundance of
245 ASV2016 and ASV4095 belonging to *H. pylori* in comparison to controls (Supplementary
246 information). As per previous reports, CeD patients with *H. pylori* gastritis were found with
247 increased numbers of intraepithelial lymphocytes in the duodenal mucosa (Villanacci et al.,
248 2006). In contrast, there are also reports which failed to reveal a relationship between *H. pylori*
249 and CeD and found that *H. pylori* presence is inversely associated CeD (Lebwohl et al., 2013).
250 However, in our study the ASV which is abundant in CeD is different than those ASVs which
251 are enriched in FDR. Our analysis included finer sequence level variation and differentiated
252 single nucleotide level difference (Callahan et al., 2016). In view of intra-genomic differences in
253 16S rRNA gene, we compared the 16S rRNA gene copies of *H. pylori* in publically available

genomes. We observed that on average the *H. pylori* genome has two copies of 16S rRNA gene and we did not observe differences between the two copies within a single genome in the V4 region investigated here (Supplementary information text page 3-7). Therefore, future studies need to focus on strain level variations and functional aspects of *H. pylori* in regards with CeD using metagenomics and functional omics. Other ASVs which showed higher abundance in CeD as compared to FDR and DC and lower abundance in FDR as compared to DC are *Megasphaera* and *Catenibacterium*. One more important observation from differential abundance results is decreased abundance of *Ruminococcus* in CeD as compared to FDR and CeD. *Ruminococcus* is considered to be a keystone taxa with influence on the microbial community and responsible for the major fraction of butyrate production in the gut (Morrison and Preston, 2016; Shetty et al., 2017; Ze et al., 2012).

Duodenal microbiota of FDR is characterized by increased abundance of ASVs related to *Actinomyces, Streptococcus, Bifidobacterium* and *Anaerostipes*. These taxa are known to possess gluten degrading enzymes, probiotic properties and ability to produce SCFA respectively (Barrangou et al., 2009; Couvigny et al., 2015; Fernandez-Feo et al., 2013; Morrison and Preston, 2016; Rivière et al., 2016). Moreover, the strain belonging to *Bifidobacterium* was reported to prevent gluten-related immunopathology in mice (McCarville et al., 2017). Higher number of these taxa in small intestine of FDRs may indicate protective role of these taxa in pre-disease state.

In comparison to duodenal biopsies, less numbers of ASVs were differentially abundant between diagnosis groups in faecal samples. This indicates more disrupted microbiome at disease site than overall gut microbiome and highlights the importance of inclusion of biopsy samples in present study.

In faecal samples, CeD showed significant enrichment of ASVs affiliated to genus *Lactobacillus* in comparison to FDR and DC. This is in line with previous study in which, higher abundance of *Lactobacillus* was observed in oral microbiome of patients with CeD (Tian et al., 2017). Moreover, there are reports stating that the certain *Lactobacillus* species degrade gliadin and increases the availability of antigenic peptides (Engstrom et al., 2015).In the present study, higher abundance of *Lactobacillus* in CeD microbiota may indicate their ability to breakdown gluten into pro-inflammatory peptides in the small intestine. Another important observation from differential abundance is the lower abundance of *Dorea* and *Akkermansia* in faecal sample of CeD. Both of these taxa are known to produce SCFAs which in turn strengthens the health of enterocytes and inhibits intestinal inflammation (Ohira et al., 2017).

Lower abundance of *Lactobacillus* and higher abundance of *Dorea* in FDR and DC in comparison to CeD indicates that the faecal microbiota of FDR is more similar to the microbiota of control samples. However, *Akkermansia* ASVs were in more abundance in control as compared to FDR.

Overall, microbiota of DC can be characterized with enriched abundance of SCFA producing core bacterial taxa, such as *Ruminococcus* and *Akkermansia* in case of biopsy and faecal samples respectively. ASVs such as *Megasphaera, Ruminococcus* and *Helicobacter* in duodenal biopsy of FDR showed higher abundance when compared with control but they are reduced in comparison with disease state CeD. Moreover, *Dorea* showed similar abundance pattern in faecal samples. In our study, the higher abundance of known pathogenic bacteria such as *Helicobacter* (well-known bacteria to be associated with intestinal inflammation) and reduced abundance of health associated bacteria such as *Akkermansia, Ruminococcus* (bacteria known for

the anti-inflammatory properties) and *Actinomyces* (a well-known gluten degrader) emerged as the characteristic of the CeD microbiota.

Through metagenome prediction method we found that the gene abundance for Xaa-pro Dipeptidase enzymes was less in CeD as compared to FDR and controls. This enzyme shows a high specificity for proline residues present in gluten and hydrolyze the peptide bond (Park et al., 2004). These observations suggest that the FDR and CeD duodenal microbiota differs in the bacterial composition and that loss or gain of specific bacteria capable of gluten degradation. This may impact gluten processing and the presentation of immunogenic gluten epitopes to the immune system in the small intestine. However, link between the predicted metagenome and gut microbiome needs to be validated with *in-vitro* enzyme assay.

The present study was conducted to investigate if the duodenal and faecal microbiotas of FDR of CeD patients are different from that of CeD and controls. Our observations from PCoA (Figure 1c and 1d) and differential abundance (Figure 2 and 3) suggest variation at lower taxonomic levels. These potential species and/or strain level variations and functional aspects need to be investigated using shotgun metagenomics and functional omics in follow-up studies.

However, metagenomic studies of biopsy samples remain a challenge because of high proportion of host DNA. Thus, predictive metagenomics using 16S rRNA gene as a practical solution was employed for biopsies. In this initial exploratory study, we investigated the gut microbiome with respect to the disease status only and future studies considering other confounding factors such as diet, body mass index age, sex, frequency and quantity of gluten intake among others will be required for a better understanding the gut microbiome in CeD and FDRs. Additionally, the control group in our study was not healthy subjects but patients with functional dyspepsia. These

subjects were used as proxy since invasive sampling procedures such as endoscopy from clinically healthy subjects is not permitted under the institutional regulations.

In summary, present study highlights the specific differences in the microbiota of FDR compared to that in patients with CeD and controls. Difference in FDR microbiota in both the faecal and duodenal biopsy samples compared to CeD suggests microbiota of FDR have unique features. Analysis of single nucleotide level variation provides a finer resolution and suggests that changes in strain level features need to be investigated in CeD. These unique features should be addressed in future mechanistic studies to understand etiopathogenesis of CeD.

Conclusions: Significant differences at ASV level suggest that specific bacterial taxa like *Helicobacter* may be important for pathogenesis of CeD. Higher abundance of beneficial bacterial taxa especially SCFA producers in controls suggest that there may be a protective role of these taxa in CeD development. Moreover, the predicted differences in gluten metabolism potential by FDR and CeD microbiota point towards the need for investigating functional capabilities of specific bacterial taxa in healthy FDR and CeD patients.

Methods

Patients and Methods

Human subjects, duodenal biopsies and faecal sample collection

A total of 62 subjects participated in this study including 23 treatment naïve patients with CeD [all HLA-DQ2/DQ8+, having high titre of anti-tissue transglutaminase antibodies (tTG Ab) and having villous abnormalities of modified Marsh grade 2 or more], 15 healthy first-degree relatives of patients with CeD [having normal titre of anti-tTG Ab and having no villous abnormalities of modified Marsh grade 0 or 1], and 24 controls (patients with Hepatitis B Virus carriers or those having functional dyspepsia; having normal titre of anti-tTG Ab and having no

villous abnormalities) (Table 1). Duodenal biopsies and faecal samples were collected from each of the above mentioned subjects at All India Institute of Medical Sciences, New Delhi, and sent to National Centre for Cell Sciences, Pune for microbiome analysis. The ethics committees of All India Institute of Medical Sciences, New Delhi, and National Centre for Cell Sciences, Pune, India approved the study. Informed and written consent was obtained from all the participants. Further details of patients and controls have been provided in the (Supplementary file 1: Table 2).

DNA extraction and 16S rRNA gene sequencing

Total DNA was extracted from duodenal biopsies using QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Germany) and faecal samples using the QIAamp fast DNA stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. We used Illumina MiSeq sequencing to determine the bacterial composition of the duodenal biopsies and faecal samples. PCR was set up in 50 µl reaction using AmpliTaq Gold PCR Master Mix (Life Technologies, USA) and with 16S rRNA V4 variable region specific bacterial primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT-3′)

Sequence processing and bacterial community analysis

Illumina Miseq platform rendered a total of 76058052 raw 16S rRNA sequence reads for the 102 faecal and biopsy samples of the diagnosis groups, with an average of 745667 ±194667 reads per sample. Adapter sequences were trimmed by using Cutadapt (1.18) tool (Martin, 2011)and trimmed reads were pooled as Fasta.gz file format for further analysis in DADA2 (v 1.6.0) pipeline (Callahan et al., 2016). In the first step reads were inspected for read quality profile, the read quality score was decreased (<30) after 240 bases for forward read and 160 bases for reverse reads. We truncated the forward reads at position 240 (trimming the last 10 nucleotides)

and reverse reads at position 160 (trimming the last 90 nucleotide). After quality filtering and removal of bases with a total of 70502947 (92.69%) high-quality reads of the 16S rRNA amplicons were obtained, with an average 691205 ± 181263 reads per sample, ranging from 325350 to 1207169 among samples (Supplementary Table 3). Finally, taxonomic assignment was done by the naive Bayesian classifier method with default setting as implemented in DADA2, against Human Intestinal 16S rRNA gene reference taxonomy database (HITdb v 1.00). Briefly, HITdb is a 16S rRNA gene database based on high-quality sequences specific for human intestinal microbiota, this database provides improved taxonomic up to the species level (Ritari et al., 2015). Unassigned chimeric and sequences of chloroplast and mitochondria were excluded from downstream analysis. Taxonomic assignment successfully mapped 6567144 ASVs (Amplicon Sequence Variants), with an average of 64383 ± 29929 ASVs per sample. Finally, from these ASVs, ASV table was constructed and the ASVs generated by the contaminants were removed by using decontam software (Davis et al., 2017) and the output ASV table was used for downstream analyses.

Microbial diversity and composition analysis was done using the R-package phyloseq (v1.22.3) (McMurdie and Holmes, 2013) and microbiome R package (v1.0.2) (Leo and Shetty, 2017). To test for similarities in bacterial communities between sample types and diagnosis groups Analysis of Similarities (ANOSIM) on Bray-Curtis distances was used. ANOSIM is a function in vegan package (v 2.4-4) to calculate significance of PCoA clustering based on the Bray-Curtis distances (Dixon, 2003).

To identify differentially abundant ASVs in pairwise comparisons between diagnosis groups we used DESeq2 (v1.18.0) (Love et al., 2014). All ASVs that were significantly (alpha = 0.01) different in abundance between the diagnosis groups were reported and were adjusted for

multiple comparisons using the Benjamini-Hochberg, false discovery rate procedure. Data was visualized using ggplot2 (v 2.2.1) in R (Hadley Wickham, 2016).

Metagenomic Imputation

Piphillin tool was used to infer metagenome from 16S rRNA ASV counts table and representative sequence of each ASV. Briefly, this tool predicts metagenomes with high accuracy by leveraging the most-current genome reference databases (Iwai et al., 2016). It uses direct nearest-neighbor matching between 16S rRNA amplicons and genomes to predict the represented genomes. Latest version (May 2017) of KEGG database and 97% of the identity cutoff was selected for the prediction. The output from Piphillin was further analyzed by STAMP statistical tool, ANOVA with post hoc Tukey-kramer test was used to identify statistically different KEGG orthologies between diagnosis groups (Parks et al., 2014).

List of abbreviations

CeD: Celiac disease, DC: Diseased controls (dyspeptic), FDR: First degree relatives. ASV: Operational taxonomic unit, rRNA: Ribosomal Ribonucleic acid, PCoA: Principal coordinates analysis.

Declarations

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Availability of data and materials

Sequence data generated in this study is available from the NCBI Sequence Read Archive within the Bioproject ID accession PRJNA385740. (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA385740) and to reproduce the analysis done in R, the R Markdown file and required data are available at https://github.com/rahulnccs/Comparison-of-Small-Gut-and-Whole-Gut-Microbiota-of-First-Degree-Relatives-with-Adult-Celiac-Disease.

Conflicts of interest: All the authors disclose no conflict of interest

Authors Contributions:

The research study was conceptualized, designed and supervised by GKM, YSS and VA. Patient recruitment, diagnosis and endoscopic examination was done by GKM; HLA testing was done by GK; biological sample collection (duodenal biopsy/stool) storage and maintenance was done by AKV, KB and AM. The extraction of genomic DNA was done by RB and PP. DKB, BPS and RCP were involved in amplicon sequencing. Bioinformatics analysis for amplicon data was done by SAS, DPD and RB. Data acquisition, data interpretation and drafting of the manuscript was done by SAS and GKM. YSS, DPD and VA critically reviewed the manuscript. All authors have read and approved the final manuscript.

436

437 **Ethics approval and consent to participate**

438 The Ethics Committees of All India Institute of Medical Sciences, New Delhi, and National
439 Centre for Cell Sciences, Pune, India approved the study. Informed and written consent was
440 obtained from all the participants.

441 Consent for publication

442 Not applicable.

443 **Conflict of Interest**

444 All authors have no conflict of interest to declare. Authors Dhinoth K. Bangarusamy, Beena P.
445 Santosh and Rajadurai C. Perumal were employed by company AgriGenome Labs Pvt Ltd.
446 Kerala, India. All other authors declare no competing interest.

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Table 1: Demographic characteristics on study subjects

Groups	No. of subjects	Age (mean±S. D.)	Gender		Sampling site		Villous abnormalities (as per Modified Marsh criteria)					HLA Haplotype			tTG Titre (mean±SD)
			M	F	S	B	0	1	3a	3b	3c	DQ2	DQ8	DQ2+ DQ8+	
CeD	23	23.4±9.5	10	13	21	16	0	0	2	7	14	22	1	0	199.9±72.1
FDR	15	31.6±10.8	6	9	15	13	15	0	0	0	0	13	0	2	4.36±2.6
DC	24	30.6±12.3	22	2	23	14	22	2	0	0	0	6	0	0	4.09±2.8

M= male, F= female, tTG= Tissue transglutaminase, S= Stool samples, B= Biopsy sample

Figure Legends

Figure 1: a. Comparison of alpha diversity between diagnosis groups in duodenal biopsies.

b. Comparison of alpha diversity between diagnosis groups in Faecal samples.

c. Principle coordinates analysis of bacterial community based on bray-curtis distance between diagnosis groups in duodenal biopsy samples.

d. Principle coordinates analysis of bacterial community based on bray-curtis distance between diagnosis groups in faecal samples

Figure 2: Comparison of differential abundance of bacterial taxa between the diagnosis groups in biopsy samples. a. Differential abundance DC vs FDR b. Differential abundance CeD vs DC c. Differential abundance CeD vs FDR. Only taxa with significant differences ($P < 0.01$) in log2 fold change are depicted.

Figure 3: Comparison of differential abundance of bacterial taxa between the diagnosis groups in faecal samples. a. Differential abundance CeD vs DC. b. Differential abundance CeD vs FDR. Only taxa with significant differences ($P < 0.01$) in average log2 fold change are depicted.

Figure 4: KO abundance for Xaa-pro dipeptidase (K01271) enzyme in faeces inferred from predicted metagenome for faecal samples. Comparison was done using ANOVA.

Supplementary file, Figure S1:

a. Principal coordinates analysis (PCoA) of bacterial community in the faecal and duodenal biopsies based on Bray–Curtis distance.

b. Comparison of alpha diversity measures between sampling sites.

Supplementary file, Figure S2: Phylum level distribution of ASVs in biopsy samples. Pairwise comparisons were done using Wilcoxon tests.

Supplementary file, Figure S3: Order level distribution of ASVs in biopsy samples. Pairwise comparisons were done using Wilcoxon tests.

Supplementary file, Figure S4: Phylum level distribution of ASVs in faecal samples. Pairwise comparisons were done using Wilcoxon tests.

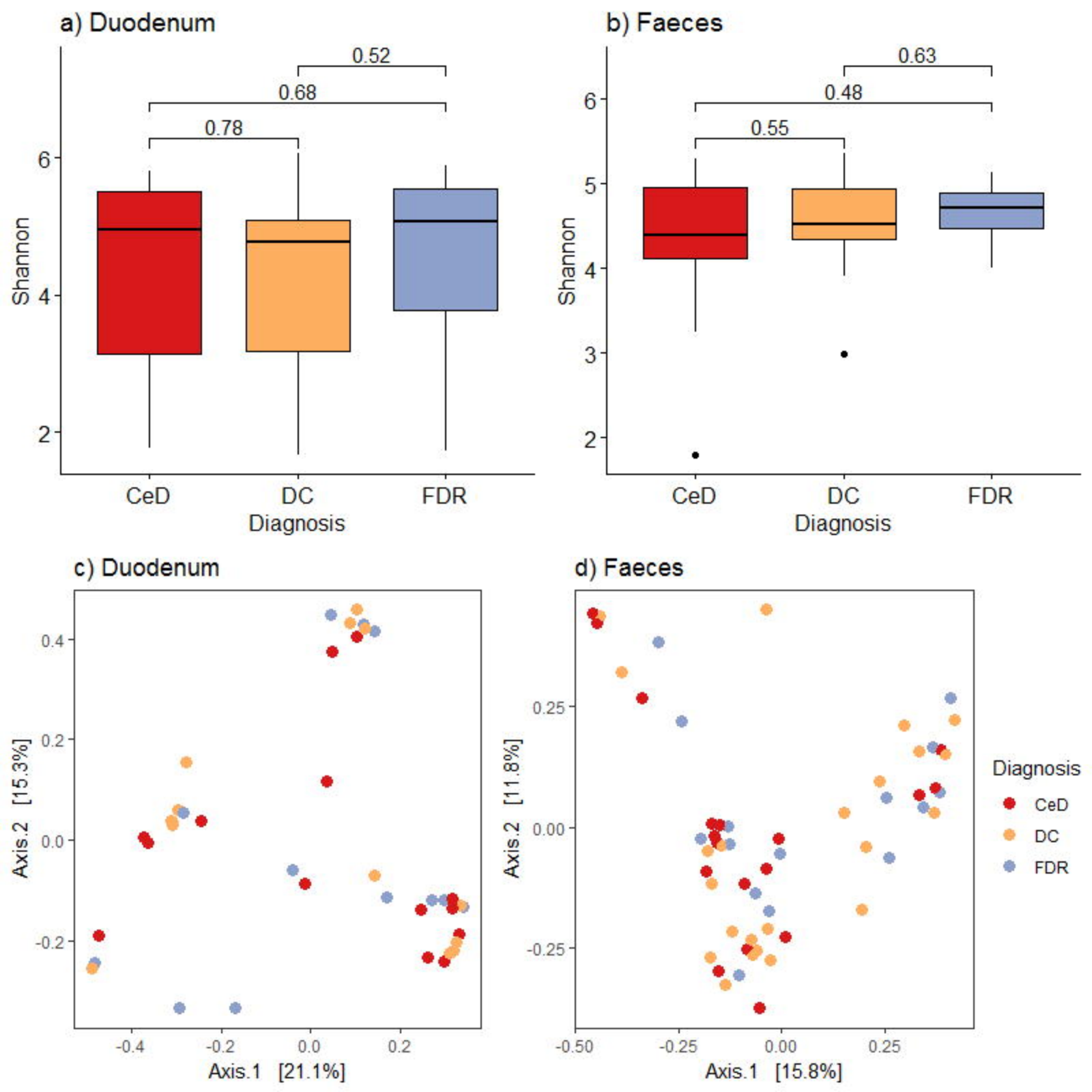
Supplementary file, Figure S5: Order level distribution of ASVs in faecal samples. Pairwise comparisons were done using Wilcoxon tests.

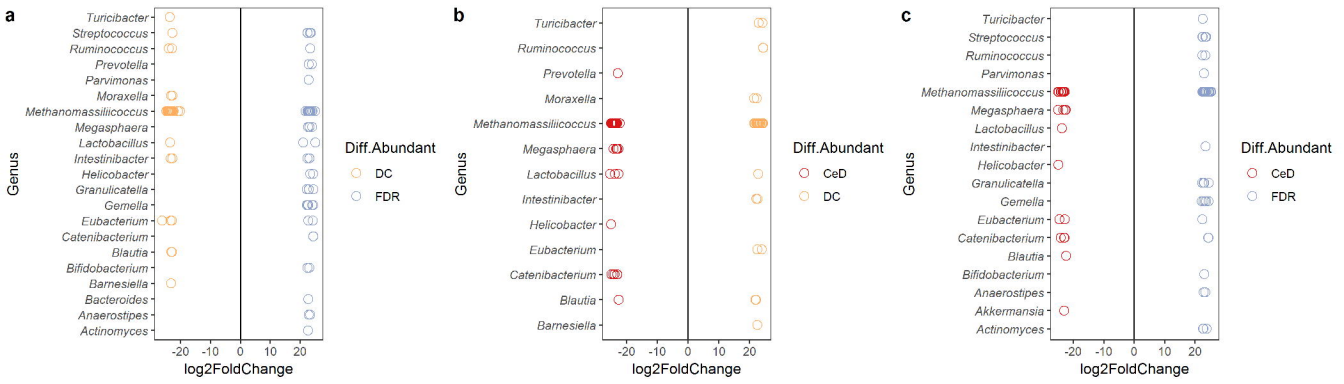
Supplementary Table 1: Significantly different KEGG orthologies (KO) between diagnosis groups

Supplementary Table 2: Details of study subjects

Supplementary Table 3: Number of reads per sample at each stage of analysis.

Supplementary Information document: Differential Abundance of Amplicon Sequence Variant of *Helicobacter*. Multiple sequence alignment was performed by CLUSTAL 2.0.11


a) Duodenum
0.52
0.68
0.78
Shannon
6
4
2
CeD
DC
FDR
Diagnosis
b) Faeces
0.63
0.48
0.55
Shannon
6
5
4
3
2
CeD
DC
FDR
Diagnosis
c) Duodenum
Axis.2 [15.3%]
0.4
0.2
0.0
-0.2
-0.4
-0.2
0.0
0.2
Axis.1 [21.1%]
d) Faeces
Axis.2 [11.8%]
0.25
0.00
-0.25
-0.50
-0.25
0.00
0.25
Axis.1 [15.8%]
Diagnosis
CeD
DC
FDR


a
Turicibacter
Streptococcus
Ruminococcus
Prevotella
Parvimonas
Moraxella
Methanomassiliicoccus
Megasphaera
Lactobacillus
Intestinibacter
Helicobacter
Granulicatella
Gemella
Eubacterium
Catenibacterium
Blautia
Bifidobacterium
Barnesiella
Bacteroides
Anaerostipes
Actinomyces
Genus
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log2FoldChange
Diff.Abundant
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b
Turicibacter
Ruminococcus
Prevotella
Moraxella
Methanomassiliicoccus
Megasphaera
Lactobacillus
Intestinibacter
Helicobacter
Eubacterium
Catenibacterium
Blautia
Barnesiella
Genus
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log2FoldChange
Diff.Abundant
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Streptococcus
Ruminococcus
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Methanomassiliicoccus
Megasphaera
Lactobacillus
Intestinibacter
Helicobacter
Granulicatella
Gemella
Eubacterium
Catenibacterium
Blautia
Bifidobacterium
Anaerostipes
Akkermansia
Actinomyces
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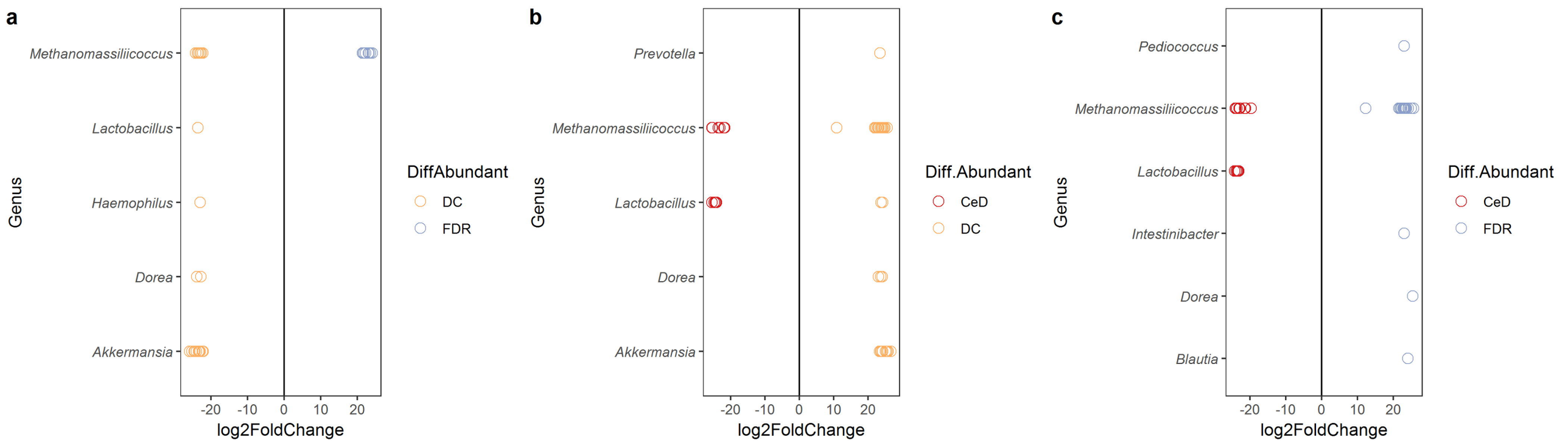
a
Methanomassiliicoccus
Lactobacillus
Haemophilus
Dorea
Akkermansia
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log2FoldChange
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Prevotella
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Pediococcus
Methanomassiliicoccus
Lactobacillus
Intestinibacter
Dorea
Blautia
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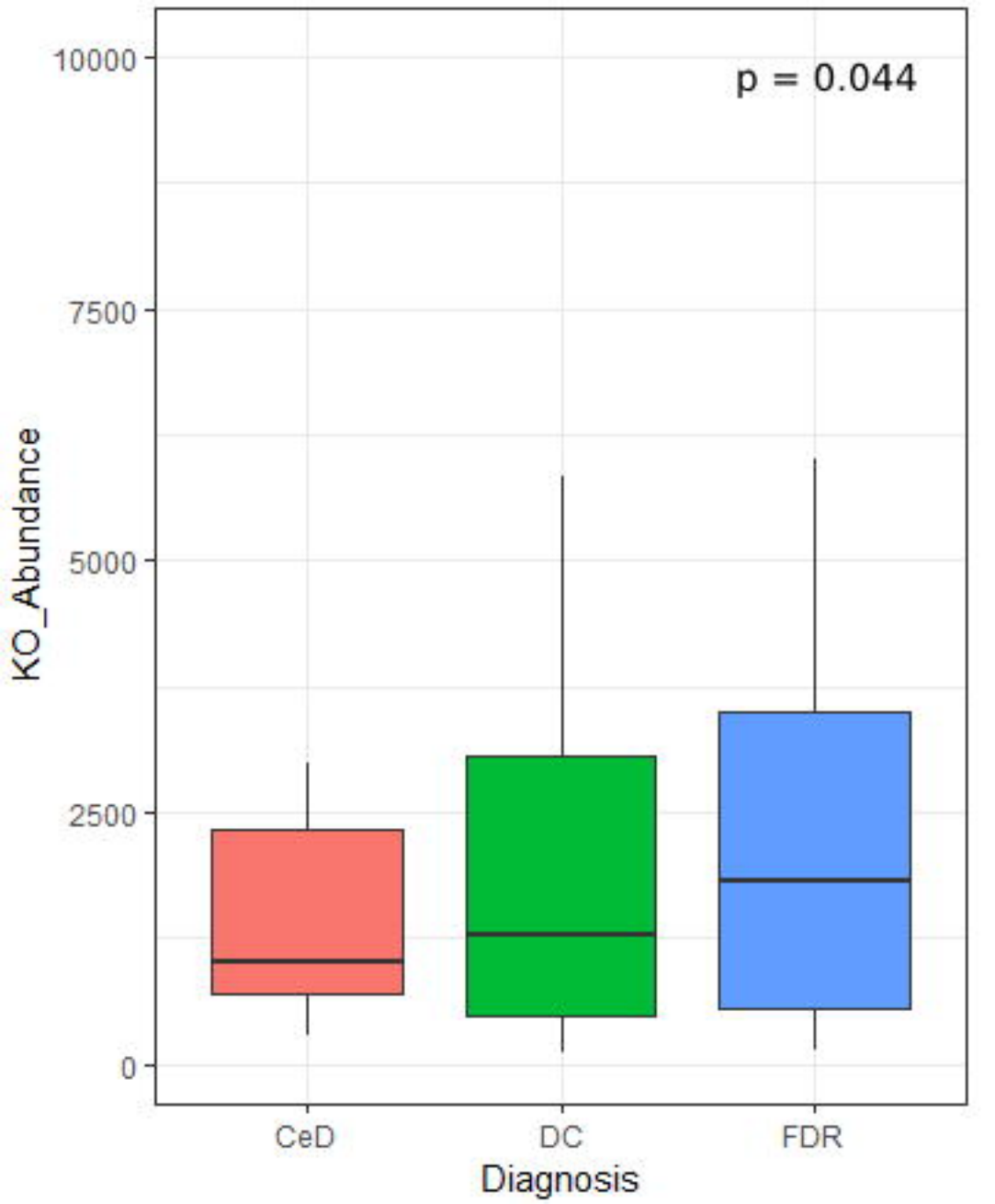
FDR


p = 0.044
10000
7500
5000
2500
0
KO_Abundance
CeD
DC
FDR
Diagnosis