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Alimentary Tract

Relationship between duodenal microbiota composition, clinical features at diagnosis, and persistent symptoms in adult Coeliac disease

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ABSTRACT

Background: Duodenal dysbiosis has been suggested to possibly influence the clinical manifestations of coeliac disease (CD), both at onset and when symptoms persist despite a gluten-free diet (GFD).
AIMS: To evaluate the relationship between duodenal microbiota composition and: i) clinical phenotype of untreated CD (UCD); ii) presence and type of persistent symptoms despite a satisfactory serological and histological response to a strict GFD.
Methods: Duodenal microbiota was analyzed by 16S rRNA sequencing and compared with i) clinical features in 12 adult UCD patients; ii) presence/absence and type of persistent symptoms (diarrhea-predominant vs. non-diarrhea predominant) in 25 adult treated coeliac patients (TCD) on a strict GFD.
Results: UCD with iron deficiency anemia (IDA) had a pro-inflammatory shift in their duodenal microbiota (reduction of *Firmicutes*, $p=0.03$; increase of *beta-Proteobacteria*, $p=0.02$) than those without IDA. TCD with persistent diarrhea showed a reduction of *Actinobacteria* ($p=0.03$) and *Rothia spp* ($p=0.046$) compared to TCD suffering from other type of persistent symptoms.
Conclusion: A distinctive duodenal microbiota profile is associated with IDA in UCD, and diarrhea-predominant persistent symptoms in TCD. Clinical interventions may include reconsidering patients presenting with IDA as a specific disease subtype, and dietary rebalancing if diarrhea persists despite histological response to a GFD.

1. Introduction

Coeliac disease (CD) is a chronic inflammatory enteropathy occurring in genetically predisposed individuals after the ingestion of gluten [1] and characterized by both a high prevalence in the general population [2] and an extremely variable clinical picture [1,3]. Mostly in adult patients, clinical manifestations vary widely in severity and include not only classical symptoms and signs of frank malabsorption [1,3], but also a wide range of extra-intestinal complaints, such as iron deficiency anemia (IDA), dermatitis herpetiformis, associated autoimmune diseases, obstetrical and gynecological disorders and neurological symptoms [1,3,4–7]. A strict lifelong gluten-free diet (GFD) is the mainstay for the treatment of CD, leading to improvement of symptoms and recovery of small bowel mucosal lesions in the vast majority of patients [1,3]. Although epidemiological data are contrasting, it has been reported that up to 30% of coeliac patients still experience persistent symptoms despite an appropriate dietary treatment [8–13].

Unraveling the possible mechanisms behind the clinical heterogeneity of CD at time of diagnosis and the persistence of symptoms despite an appropriate dietary treatment is a hard issue to be addressed [10–12].

Growing interest has been devoted to the possible contributing role of alterations of the intestinal microbiota composition (ie. dysbiosis) in the pathogenesis and clinical manifestations of CD [14]. So far, however, only eight studies have investigated the intestinal microbiota composition in adult coeliac patients (Table 1) [15–22]. Among these, only one Finnish paper reported a correlation between duodenal microbiota composition and clinical manifestations of CD, and another paper by the same group described a relationship between duodenal dysbiosis and persistent symptoms in coeliac patients on a strict GFD [17,18].

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Table 1
Papers that have evaluated microbiota composition in adult coeliac disease.

Paper	Study population	Samples	Methods	Main findings	Altered phylum	Altered groups
Nistal, 2012, Spain [15]	10 untreated CD 11 treated CD on a GFD 11 non-CD controls	Feces	PCR-DGGE and gas-liquid chromatography of SCFAs	Changes in microbiota composition in untreated CD; restoration of "normal" microbiota in treated CD	↓ *Lactobacillus* and *Bifidobacterium* diversity in treated CD ↑ *Bifidobacterium bifidum* in untreated CD vs. healthy controls	
Nistal, 2012, Spain [16]	8 untreated CD children 5 healthy children 15 untreated CD adults 15 healthy adults	Duodenal biopsies	16SrRNA sequencing	Bacterial richness significantly lower in children than adults Different microbiota composition in adult with untreated CD vs. CD on a GFD		
Wacklin, 2013, Finland [17]	33 untreated CD 18 non-CD controls	Duodenal biopsies	Nested PCR-DGGE and 16SrRNA	Changes in microbiota composition and diversity according to clinical pattern of CD	↑ *Proteobacteria* in coeliac patients with GI symptoms ↑ microbial diversity and richness in DH patients than coeliac with GI symptoms or asymptomatic	*Acinetobacter* and *Neisseria* more abundant in coeliac patients with GI symptoms *Streptococcus* and *Prevotella* more abundant in DH and non-CD controls
Wacklin, 2014, Finland [18]	18 treated CD patients with histological response but persisting symptoms despite a GFD vs. 18 treated CD patients with good histological and clinical response to a GFD	Duodenal biopsies	16SrRNA pyrosequencing	↓ Microbial richness and significant duodenal microbiota composition in treated CD with persisting symptoms	In treated CD with persisting symptoms: ↑ *Proteobacteria* ↓ *Firmicutes* ↓ *Bacteroidetes*	In treated CD with persisting symptoms: ↓ *Prevotella* ↓ *Uncl. Lactobacillales* ↓ *Uncl. Lachnospiraceae* ↓ *Megasphaera* ↓ *Uncl. Veillonellaceae* ↓ *Bergeriella* ↓ *Uncl. Firmicutes*
D'Argenio, 2016, Italy [19]	20 untreated CD 6 treated CD on a GFD 15 non-CD controls	Duodenal biopsies	16SrRNA pyrosequencing	Changes in microbiota composition	In untreated CD: ↑ *Proteobacteria* ↓ *Firmicutes* ↓ *Actinobacteria*	In untreated CD: ↑ *Neisseria Flavescens*
Nistal, 2016, Spain [20]	9 untreated CD 9 non-CD controls	Duodenal biopsies	16SrRNA pyrosequencing	Bacterial richness and diversity were higher in non-CD controls, but the differences were not statistically significant		
Brodke, 2019, India [21]	23 CD patients 15 first degree relatives of CD patients 24 controls	Duodenal biopsies and feces	16S rRNA gene sequencing	Reduced ability of gluten degradation by fecal microbiota in CD patients	In CD vs. first degree relatives: ↑ *Megasphaera* ↑ *Helicobacter* In CD vs. controls: ↓ *Akkermasia* ↑ *Dorea*	
Panelli, 2020, Italy [22]	13 active CD 6 potential CD 29 treated CD 4 refractory CD 31 non-CD controls	Saliva, feces, duodenal biopsies	16SrRNA metagenomic approach	Changes in a- and b-diversity and microbiota composition Salivary findings mirrored the mucosal results better than feces Expansion of pathobiontic species anticipates villous atrophy and is maximum in refractory CD	In untreated CD: ↑ *Proteobacteria* ↓ *Firmicutes* ↓ *Bacteroidetes* In refractory CD: ↓ *Fusobacteriaceae* ↓ *Lechnospioraceae*	In untreated CD: ↑*Neisseria*

CD: coeliac disease; DH: dermatitis herpetiformis; GFD: gluten-free diet; SCFAs: short chain fatty acids; GI: gastro-intestinal.

The aim of the present study was to evaluate whether distinct duodenal microbiota signatures can play a role in determining the clinical phenotype of adult CD and whether dysbiosis may be associated with persistence of symptoms in coeliac patients with a satisfactory serological and histological response to a long-term strict GFD.

2. Patients and methods

2.1. Patients and study design

This is a single-center prospective study, which primarily aims to investigate the possible relationship between duodenal microbiota composition and: i) the clinical and histological phenotype of untreated CD; ii) the presence and type of persistent symptoms despite a satisfactory serological and histological response to a strict GFD.

Recruitment of patients and sampling were prospectively carried out at the Gastroenterology Department of the I.R.C.C.S. Policlinico San Matteo Foundation (Pavia, Italy) between November 2015 and February 2018.

Subjects consecutively enrolled in this study included adult patients (age $\geq$ 18 years) affected by untreated CD on a gluten-containing diet (GCD) and treated coeliac patients (TCD) on a strict long-term GFD.

Exclusion criteria for this study included presence of severe comorbidities, such as psychiatric disorders, organ failure, concurrent infections or immunodeficiencies, malignancy, history of intestinal

surgery, as well as recent (within 4 weeks) or current use of medications possibly affecting bowel function and/or microbiota composition, such as antibiotics, probiotics, opioids, non-steroidal anti-inflammatory drugs, proton pump inhibitors, laxatives, steroids, anti-diarrhoeal drugs, iron, folate and vitamin supplements. Patients affected by seronegative CD [23] and refractory/complicated CD [12] were also excluded.

2.2. Clinical, histological and dietary evaluation

At time of enrolment in the study, all coeliac patients underwent thorough clinical assessment, laboratory testing for Human Leukocyte Antigen (HLA), coeliac serology and upper GI endoscopy with duodenal biopsies on the same day they attended the clinic. Patients on a GCD who had positive IgA endomysial antibodies (EmA), normal IgA levels, and a certain degree of mucosal abnormalities on duodenal biopsies were considered as affected by untreated CD (UCD). Presenting symptoms, biochemical alterations and severity of duodenal histological lesions according to the Corazza-Villanacci classification [24] at time of diagnosis of CD were collected. With respect to the histology at diagnosis, the UCD group included both patients with active CD (i.e. positive EmA and villous atrophy on duodenal biopsy, Corazza-Villanacci grade B) and patients with potential CD (i.e. positive EmA and normal duodenal architecture, Corazza-Villanacci grade A) [3].

Patients with biopsy proven CD on a strict GFD were enrolled as treated coeliac patients (TCD). For this group, adherence to a GFD was evaluated by means of duodenal biopsy, EmA and a standard five-level score we previously developed and validated [25]. All TCD patients considered in this study were on a strict GFD (score 3 or 4 of the questionnaire we previously developed [25]), had a fully satisfactory serological and histological response to a GFD (negative EmA and absence of villous atrophy on follow-up duodenal biopsy). Persistent gastro-intestinal symptoms and/or biochemical abnormalities despite being on a GFD were collected for all TCD patients on a long-term GFD. TCD patients were then divided into a 'persistent symptoms' group and a 'no symptoms' group for the purpose of the microbiota analysis. The 'persistent symptoms' group was further subdivided according to the predominant type of persistent symptom (diarrhea-predominant vs. non-diarrhea-predominant). We specify that the groups of UCD and TCD patients are separate (i.e. patients in the TCD group were not previously analyzed as UCD).

2.2.1. Small bowel mucosal samples

Six perendoscopic biopsy specimens were taken from the second duodenal portion during upper gastro-intestinal endoscopy performed under mild sedation. Four correctly oriented specimens were formalin-fixed and paraffin-embedded for traditional haematoxilin and eosin histology and immunohistochemistry, while two specimens were snap-frozen in liquid nitrogen and stored at −80 °C until use for microbial DNA extraction.

2.3. Laboratory tests and coeliac disease genetics

Coeliac serology. IgA EmA were detected on monkey/jejunum esophagus sections using an indirect immunofluorescence kit (ANOVA Diagnostic, San Diego, USA). We specify that in our center we do not test patients routinely for tTG, as both sensitivity and specificity of EMA and tTG are very similar and satisfactory, as previously described [26].

HLA typing. Patients were typed for HLA class II genomic polymorphisms at the high-resolution level by means of sequences specific primers-polymerase chain reaction (PCR-SSP) and/or sequence-specific oligonucleotides primed -polymerase chain reaction (PCR-SSO) [27]. The DNA was extracted from peripheral blood samples using the Wizard genomic DNA Purification kit (Maxwell 16, Promega Instrument; Madison, WI, USA) according to the manufacturer's protocol. The polymorphism of the HLA-DQA1 and DQB1 genes was analyzed using commercial kits (Olerup SSP AB, Stockholm; Sweden - One Lambda Inc., Canoga Park, CA, USA). Amplified products were visualized on 2% agarose gels stained with 0.5 mg/mL ethidium bromide, using the E-Gel precast Agarose Electrophoresis System (Invitrogen Life Technologies, PA4 9RF Paisley, UK). HLA-DQ2 positive patients carried the HLA-DQ2.5 (encoded by the alleles DQA1*05 DQB1*0201) or the HLA-DQ2.2 (encoded by the alleles DQA1*0201 DQB1*0202) molecules, while HLA-DQ8 positive patients carried the HLA-DQ8 molecules (encoded by the alleles DQA1*03 DQB1*0302) [28].

2.3.1. Extraction and quantification of DNA

DNA was extracted from snap-frozen duodenal mucosal samples by using commercial kits (Dneasy Blood&Tissue Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions to assure an unbiased representation of the profiles of both $Gram^+$ and $Gram^-$ bacteria [29]. The DNA concentration of extracted samples was assessed fluorometrically on a Qubit 2.0 instrument (Thermo Fisher Scientific; Waltham, MA, USA).

2.3.2. Production of 16S rRNA amplicons (V3-V4 regions), sequencing and taxonomic assignment

For amplicon production, the V3-V4 hypervariable regions of the prokariotic 16S rRNA gene were targeted to select bacterial DNA from the total DNA extract according a method previously validated [30]. PCR was performed in a 50 µL volume containing template DNA, 1xHiFi HotStart Ready Mix (Kapa Biosystems; Wilmington, MA), and 0.5 µM of each primer. The cycling program, performed on a MJ Mini thermal cycler (Biorad corp.; Hercules, CA, USA), included an initial denaturation cycle (95 °C for 3 min), followed by 25 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension (72 °C for 5 min). Clean-up of amplicons was performed using Agencourt AMPure XP SPRI magnetic beads (ThermoFisher Scientific). Illumina sequencing libraries were finally constructed through the link of indexes (Nextera XT Index Kit, Illumina; San Diego, CA, USA), quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific), normalized and pooled. Libraries were subjected to paired-end sequencing (2×300 bp format) on an Illumina MiSeq platform at BMR Genomics (Padua, Italy). The microbial composition and diversity were assessed according to an *ad hoc* procedure, as previously reported [21]. Filtered reads were organized into operational taxonomic units (OTUs) at 97% nucleotide similarity and assigned to their taxonomy through the Greengenes 16S rRNA bacterial database (version 13.8) [31].

2.3.3. Statistics

Biopsy samples that were homogeneous with respect to each population subtype were considered. Only taxa showing normal distribution were considered in each population subtype. Shapiro-Wilk test was used for normalizing data before proceeding to statistical comparisons by means of Student's *t*-test for independent data. A statistical p-value < 0.05 was considered significant. R software (R version 3.6.3, 2020–02–29) was used for computation. Shannon index was used to compare biodiversity across TCD patients with and without persistent symptoms.

2.3.4. Ethics

This study was conducted in accordance with the Declaration of Helsinki (6^{th} revision, 2008) and all the procedures involving collection and processing of human biological samples were approved by the local Ethics Committee (protocol number 20,150,003,822, procedure number 20,150,019,762). All the patients enrolled in this study gave written informed consent to participate.

Table 2
Demographic and clinical features of celiac patients enrolled in the study.

	ACD $N=12$	TCD (on a strict GFD) $N=25$		
	4 PCD + 8 UCD	No symptoms $N=18$	Persistent diarrhea $N=3$	Other persistent symptoms $N=4$
Demographics				
Gender, Female	7	12	3	3
Age at enrolment (years, mean ± SD)	43 ± 10	33 ± 11	40 ± 12	43 ± 17
Median time on a GFD (mean, SD)	NA	4.6 ± 0.7	7.5 ± 2.1	4.5 ± 3.0
Median BMI (25^{th}–75^{th})	21.6 (18.9–25.9)	20.5 (19.2–23.9)	Patient 1: 20.0 Patient 2: 20.5 Patient 3: 32.9	19.95 (19.3–20.6)
Clinical presentation at time of diagnosis of CD				
Diarrhea	1	0	3	0
WL	3	0	0	0
IDA	5	5	1	1
Dyspepsia	0	0	0	1
GORD	0	0	0	1
Autoimmunity*	4	4	0	3
First degree Family history of CD	2	3	1	0
Histology				
Atrophic (Corazza-Villancacci B grade)	8	0	0	0
Non atrophic (Corazza-Villanacci A grade)	4	18	3	4
Genetics				
**HLA DQ2^{+ve}				
homozygous	2	3	2	2
heterozygous	10	9	1	0
HLA DQ8^{+ve}	0	2	0	0
heterozygous	0	2	0	0

ACD: active celiac disease; CD: celiac disease, PCD: potential celiac disease (on a gluten-containing diet); TCD: treated celiac disease; UCD: untreated celiac disease; BMI: body mass index; GFD: gluten-free diet; GORD: gastroesophageal reflux disease; IDA: iron-deficiency anemia; WL: weight loss; SD: standard deviation; NA: not assessed.
**Autoimmunity includes patients with dermatitis herpetiformis;.*
***HLA typing was not available in seven treated celiac patients (five in the group without persistent symptoms and two in the group with further persistent symptoms). In one treated celiac patient without persistent symptoms, HLA was DQ2/DQ8 heterozygous.*

3. Results

Altogether, 39 coeliac patients were enrolled in this study. Duodenal microbiota profiling was successful in 37/39 patients. These included 12 patients affected by UCD on a GCD and 25 patients with TCD on a long-term strict GFD (median time on a GFD prior to recruitment three years, 25^{th}–75^{th}, two-seven years). Seven out of 25 TCD patients had persistent symptoms despite a satisfactory histological and serological response to the GFD. Of these, three had diarrhea, two gastro-oesophageal reflux disease, one dyspepsia and one bloating. Demographic and clinical features of all the patients enrolled in the study are shown in Table 2.

A total of 5,327,971 high-quality reads were obtained for mucosal samples. The reads for each sample were higher than 40.000, sufficient for identifying all biodiversity.

3.1. Relationship between clinical and histological features and duodenal microbiota in untreated CD

Analysis of duodenal microbiota relative abundance in the 12 UCD patients showed no differences according to the degree of histological lesions at taxa, phyla, class and genera level. As a consequence, no differences between eight patients with active CD (i.e. villous atrophy, Corazza Villanacci B) and four affected by potential CD (i.e. absence of villous atrophy, Corazza-Villanacci A) were found. Thus, these two groups were considered together for the purpose of the analysis aimed at evaluating the relationship between symptoms at diagnosis and duodenal microbiota profile. In this regard, average abundance of bacterial taxa differed significantly between patients presenting with IDA at diagnosis and those without. More precisely, the relative abundance of taxon *Streptococcus_unclassified* was higher in patients without IDA than in those with IDA (23.01% vs. 11.46%, $p=0.02$). No difference was found when weight loss, diarrhea, dyspepsia, family history of CD or associated autoimmune conditions were considered.

3.2. Average bacterial abundances at taxonomic levels

At phylum level, coeliac patients with IDA at diagnosis had a lower relative abundance of *Firmicutes* (24.28% vs. 40.57%, $p=0.03$) and a higher relative abundance of *Proteobacteria* (41.11% vs. 20.76%) as compared to non-anemic patients. This difference in the microbiota composition between anemic and non-anemic patients was mirrored at class level for *Betaproteobacteria* (30.05% in patients with IDA vs. 10.46% in those without IDA, $p=0.02$) and at genera level for *Streptococcus* (11.47% in patients with IDA vs. 23.14% in those without IDA, $p=0.02$). No significant differences were found at species level. Fig. 1 shows the differences in the average bacterial abundances at taxomic levels between untreated coeliac patients with and without IDA at diagnosis.

3.3. Relationship between persistent symptoms and duodenal microbiota in coeliac patients on a gluten-free diet

Duodenal microbiota composition did not differ significantly at taxonomic level between TCD suffering from persistent symptoms ($n=7$) and those without persistent symptoms ($n=18$).

Taking into account the great clinical heterogeneity of TCD patients with persistent symptoms, we furtherly divided them according to the predominant type of persistent symptoms into two groups: 'diarrhea-predominant' persistent symptoms ($n=3$) and non-diarrhea predominant persistent symptoms ($n=4$). We then compared duodenal microbiota composition across these three groups of TCD patients (complete clinical response to a GFD

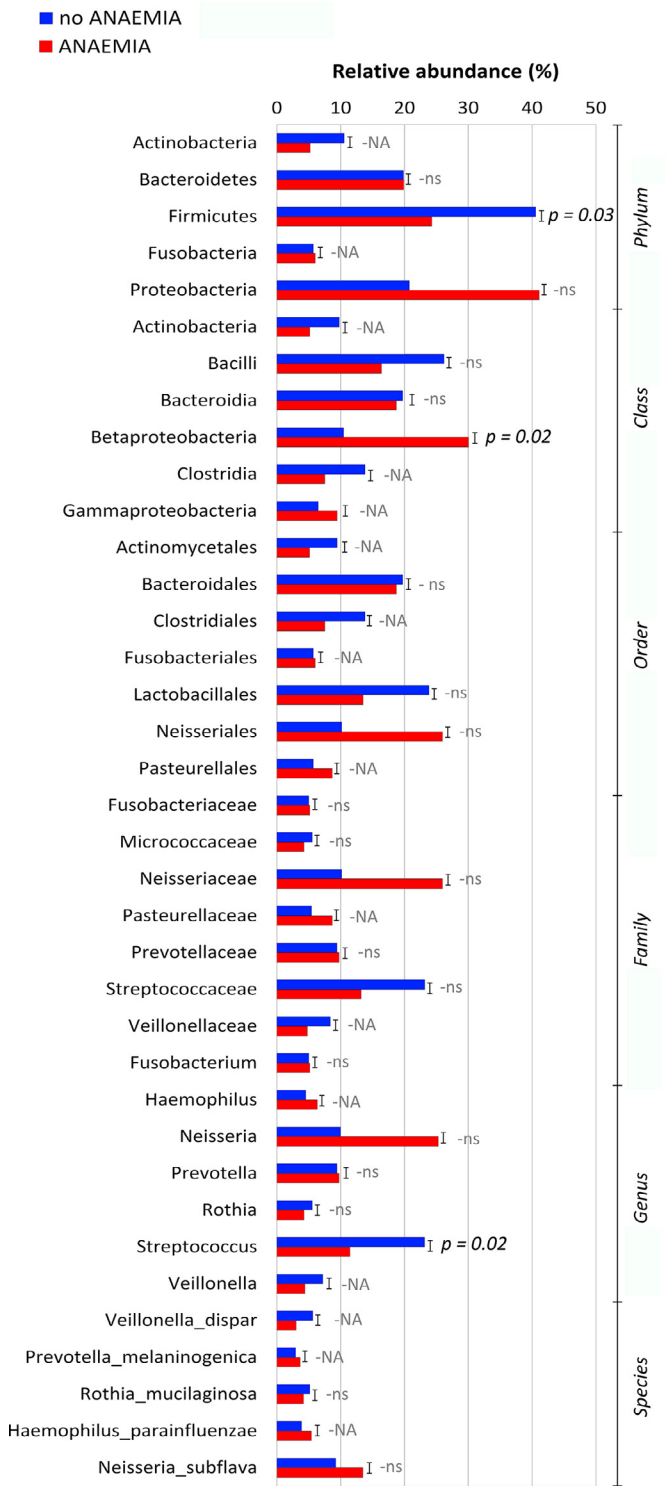

Fig. 1. Differences in the average bacterial abundances at phyla, class and genera levels in untreated coeliac patients with and without anemia at diagnosis.
Dark gray columns: patients with anemia; light gray columns: patients without anemia; NA: not applicable; ns: not significant.

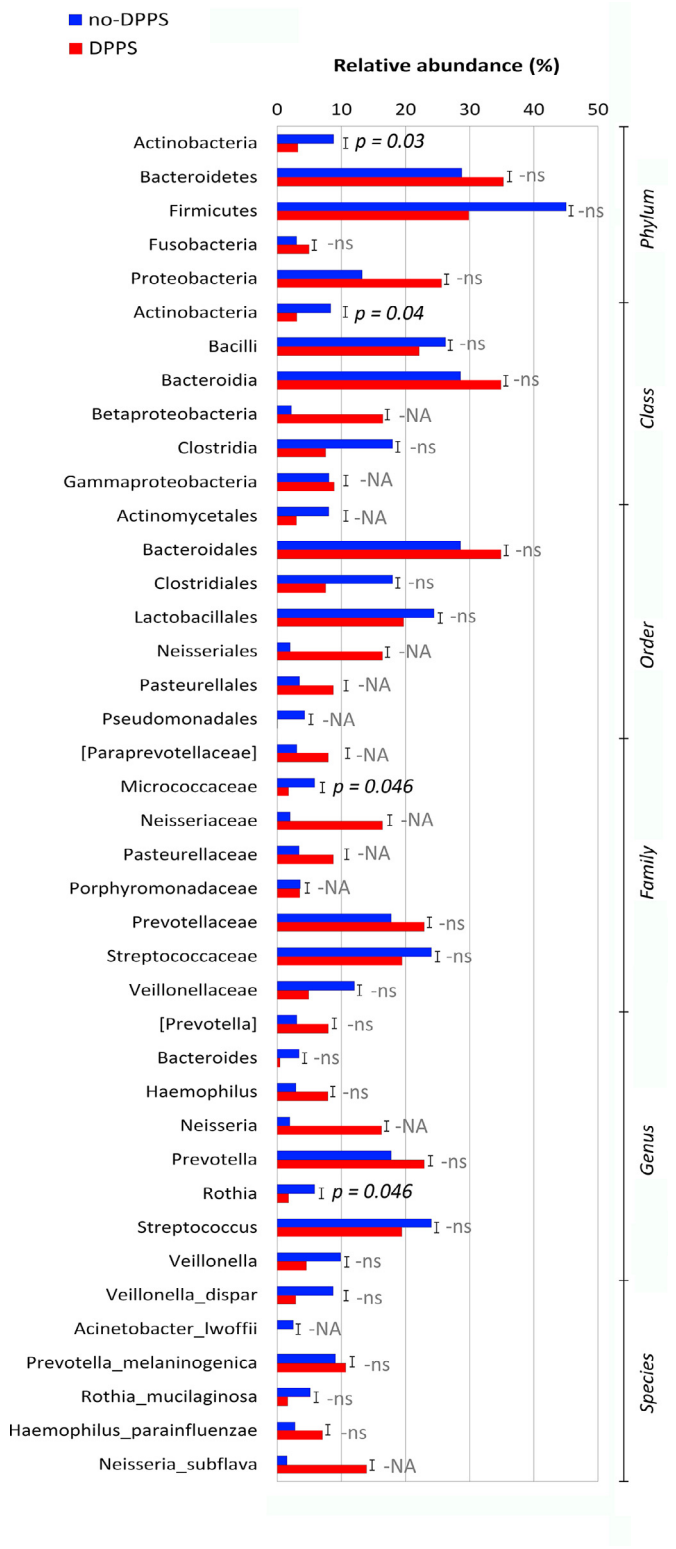

Fig. 2. Differences in the average bacterial abundances at phyla, class and genera levels between treated coeliac patients with diarrhea-predominant persistent symptoms and those with non-diarrhea-predominant persistent symptoms.
DPPS: diarrhea predominant persistent symptoms; no-DPPS: non-diarrhea predominant persistent symptoms; NA: not applicable; ns: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vs. diarrhea-predominant persistent symptoms vs. non-diarrhea predominant persistent symptoms). Table 3 shows the average abundances of bacterial taxa which significantly differed among groups. Patients with diarrhea-predominant persistent symptoms had a marked reduction of *Rothia dentocariosa* ($p < 0.01$) and *Lachnospira_unclassified* ($p = 0.03$) when compared to TCD patients with satisfactory clinical response to a GFD. The latter had a

Table 3
Relative abundances of bacterial taxa that significantly differ between treated coeliac patients with complete clinical response to a gluten-free diet and treated coeliac patients with different types of persistent symptoms despite a gluten-free diet.

Taxon	Group 1TCD complete clinical response (%)($n=18$)	Group 2TCDwith DPPS (%)($n=3$)	Group 3TCDwith no-DPPS (%)($n=4$)	Group 1vs.Group 2p-value	Group 1 vs.Group 3p-value	Group 2vs.Group 3p-value
Rothia_dentocariosa	0.24	0.06	–	<0.01	–	–
Lachnospiraceae_unclassified	0.07	0.02	–	0.03	–	–
[Prevotella]_unclassified	4.43	–	1.88	–	0.04	–
Haemophilus_unclassified	0.46	–	0.13	–	<0.01	–
Mogibacterium_unclassified	0.06	–	0.02	–	0.03	–

DPPS: diarrhea predominant persistent symptoms; no-DPPS: non-diarrhea predominant persistent symptoms; TCD: treated coeliac disease; GFD: gluten-free diet.

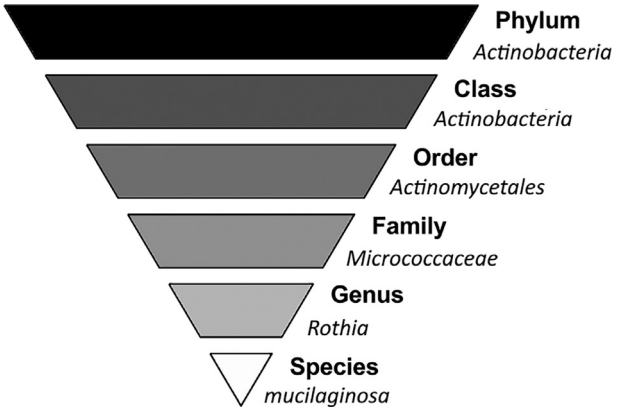

Fig. 3. Taxonomic origin of Rothia mucilaginosa.

significantly higher abundance of *Prevotella_unclassified* ($p=0.04$), *Haemophilus_unclassified* ($p<0.01$) and *Mogibacterium_unclassified* ($p=0.03$). No substantial differences were found between the two groups of TCD patients with persistent symptoms. By contrast, when moving to the taxonomic level, a significant difference emerged from the comparison between TCD patients with diarrhea-predominant-persistent symptoms and those with non-diarrhea-predominant symptoms. More specifically, TCD patients with diarrhea-predominant persistent symptoms had a marked reduction of *Actinobacteria* at phylum level (3.23% vs. 8.80%, $p=0.03$), that was paralleled by a reduction of *Micrococcaceae* at family level (1.77% vs. 5.84%, $p=0.046$) and by a reduction of *Rothia* at genera level (1.76% vs. 5.84%, $p=0.046$). Fig. 2 shows the differences in the average bacterial abundances at phyla, class and genera level between TCD patients with diarrhea-predominant persistent symptoms and those with non-diarrhea-predominant persistent symptoms. Finally, although not significant, at species level *Rothia mucilaginosa* had a different relative abundance among groups (1.65% in TCD with diarrhea-predominant persistent symptoms vs. 5.15% in TCD with non-diarrhea predominant persisting symptoms). Fig. 3 summarises the taxonomic origin of *Rothia mucillaginosa* found in TCD patients with diarrhea predominant persistent symptoms. The analysis of biodiversity by means of the Shannon index did not reveal significant differences between TCD with and without persistent symptoms.

4. Discussion

Perturbations of duodenal microbiota composition have been suggested as playing a role in the development of clinical manifestations of CD, both at onset, and when symptoms persist despite a strict and long-term GFD [14,17,18]. However, results of the studies on adult coeliac patients are difficult to compare because of differences in the enrolled populations and methods for assessment of microbiota composition [15–22] (summarised in Table 1). In the present study, we found that duodenal microbiota composition differs according to the clinical phenotype of adult CD and that it likely plays a role in patients suffering from diarrhea predominant persistent symptoms despite a strict GFD.

We found that coeliac patients presenting with IDA at diagnosis had a distinctive pro-inflammatory duodenal microbiota profile characterised by a low relative abundance of Firmicutes and a high relative abundance of Beta-Proteobacteria, which is not evident in non-anemic patients (including those presenting with diarrhea and/or weight loss). We also found that *Streptococcus_unclassified* is less abundant in duodenal specimens of patients with IDA. A previous study by Wacklin et al. showed that the duodenal microbiota was similar between coeliac patients with gastro-intestinal symptoms and those with anemia at diagnosis, even if these two groups differed from patients with dermatitis herpetiformis [17]. Interestingly, a reduction of *Firmicutes* and *Bacteroidetes*, was found also in patients with inflammatory bowel diseases [32]. A mouse model of intestinal inflammation showing that hepcidin, a key regulator of iron homeostasis in mammals, plays a crucial role in tissue repairing through its interaction with intestinal microbiota, has recently been reported [33]. This study showed that dendritic cell-derived hepcidin is induced by microbial stimulation and by acting on ferroportin-expressing phagocytes, it promotes local iron sequestration, which regulates the microbiota and consequently facilitates intestinal repair [33]. Although similar studies in humans are lacking, it would be intriguing to hypothesize such an interaction also in CD presenting with IDA at diagnosis. In light of our findings, we may hypothesize that coeliac patients presenting with IDA represent a peculiar phenotype of CD, possibly characterised by a more severe clinical picture.

Finally, we did not find a substantial difference in the duodenal microbiota composition between patients with a complete clinical response to a GFD and those with persistent symptoms. This result contrasts with the work by Wacklin et al., which reported a reduced microbial richness and dysbiosis characterised by an increase in *Proteobacteria*, and reduction in both *Firmicutes* and *Bacteroidetes* in CD with persistent symptoms despite a strict GFD [18]. However, in this paper TCD patients with persistent symptoms were very homogeneous, and all but two had symptoms suggestive of diarrhea predominant irritable bowel syndrome. This represents only a possible scenario among the different types of persistent symptoms that coeliac patients may experience [8–13]. This is the reason why in our study two groups of patients with persistent symptoms were considered, i.e. diarrhea-predominant and non-diarrhea predominant, which were indeed characterised by different duodenal microbiota profiles. Patients with diarrhea-predominant persistent symptoms showed a reduction of *Actinobacteria* at Phyla level and this was paralleled of by a reduction of *Micrococcaceae* and by the lower abundance of *Rothia* at genus level. It is noteworthy that *Rothia* species have been shown to have high gluten degrading activity *in vitro* [34, 35]. These microorganisms are commonly harboured in the oral cavity, where the abundance of dietary gluten peptides represents a major driving-factor for microbial colonization. It is certainly tempting to speculate that the reduction of *Rothia ssps* in TCD patients suffering from diarrhea

despite a GFD may be due to the fact that their GFD is indeed very strict. This would be in agreement with a previous study by our group showing that patients with persistent symptoms had better adherence to a GFD, thus possibly pointing to a causal role of other dietetic factors for their symptoms (Fermentable Oligo-, Di- and Mono- saccharides And Polyols-FODMAPs, and high consumption of gluten-free packaged and processed foods) [36].

Another aspect worthy of note is that the duodenal microbiota profile of patients with a complete clinical response to a GFD was dominated by commensal species such as *Haemophilus*, *Prevotella* and *Mogibacterium*. Although we did not plan to compare the duodenal microbiota profile of TCD patients with that of non-coeliac controls, it is possible that in coeliac patients a complete resolution of clinical, histological and microbiological alterations occurred upon a strict GFD. Interestingly, this is the first time that *Mogibacterium* has been described in CD. According to the literature, a higher abundance of *Mogibacterium* was reported in patients with Graves's disease [37], colorectal cancer [38], and in individuals affected by geographic tongue [39]. Of course, it is hard to compare these results with ours, given the wide heterogeneity of these conditions and the different methods used to assess microbiota composition. Our finding that *Prevotella* was more abundant in TCD patients with complete clinical response to a GFD is in line with the paper by Wacklin et al. reporting a higher abundance of *Prevotella* in TCD without symptoms [18] and with the work by Nistal et al. showing that number of sequences of *Prevotella* were more abundant in TCD and healthy controls than in untreated CD [16].

The main limitations of our work are the small sample size of the study population, the single center design and the strictness of the exclusion criteria needed to avoid possible confounding variables (comorbidities, medications and diets possibly affecting the microbiota composition). Therefore, we were not able to include patients affected by a silent form of CD and to confirm the influence of HLA-DQ2 and DQ8 haplotypes on duodenal microbiota composition, as previously suggested in pediatric studies [40,41,42]. In addition, although potential CD and active CD are two distinct clinical entities, we pooled them together in the UCD group because of the results of our previous study showing similarities in the microbiota composition between these two groups [22]. This certainly might have affected the possibility to find a difference according to the severity of the histological lesions.

Another limitation is linked to the amplicon metagenomics itself, which relies on the sequencing of one or more variable regions of the highly conserved gene coding for the small subunit ribosomal RNA (16S rRNA). While it is a widely-used approach for profiling bacterial microbiota, it must address, among others, the limited taxonomic and functional information contained in short sequences. This is partly overcome by the more expensive metagenomic sequencing, in which whole genomes from virtually every member of the bacterial community are sequenced in a shotgun manner [43]. The ability of constructing a "catalog of genes" of the bacterial community and the possibility of a strain-level identification of bacteria together with the functional information provided on genomes are particularly relevant to the issues discussed in this paper. It is predictable that, with the lowering of prices, future studies (comprised large-scale ones) will use more and more often this approach, also thanks to recent observations showing that only low coverage is needed in shotgun sequencing if the goal is to understand which species and functions are present in given groups or pathologies, with per-sample costs as 16S sequencing (so-called "shallow metagenomics") [44].

In conclusion, our results, although on a very limited sample size, confirm that a certain degree of duodenal dysbiosis is associated with the clinical manifestations of adult CD, both at time of diagnosis and in patients suffering from diarrhea-predominant persistent symptoms despite a strict GFD. Implications for clinical practice may include reconsidering patients with IDA at diagnosis as a specific disease subtype with a specific pro-inflammatory profile, and expert dietary counselling as first line intervention in coeliac patients with persistent diarrhea despite a good histological response to a GFD.

Declaration of Competing Interests

None to declare

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