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# Characterization of the “gut microbiota-immunity axis” and microbial lipid metabolites in atrophic and potential celiac disease

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**Introduction:** Potential celiac disease (pCD) is characterized by genetic predisposition, positive anti-endomysial and anti-tissue transglutaminase antibodies, but a normal or almost normal jejunal mucosa (e.g., minor histological abnormalities without villous atrophy). To gain further insights into basic mechanisms involved in the development of intestinal villous atrophy, we evaluated and compared the microbial, lipid, and immunological signatures of pCD and atrophic CD (aCD).

**Materials and methods:** This study included 17 aCD patients, 10 pCD patients, and 12 healthy controls (HC). Serum samples from all participants were collected to analyze free fatty acids (FFAs). Duodenal mucosa samples of aCD and pCD patients were taken to evaluate histology, tissue microbiota composition, and mucosal immune response.

**Results:** We found no significant differences in the mucosa-associated microbiota composition of pCD and aCD patients. On the other hand, in pCD patients, the overall abundance of serum FFAs showed relevant and significant differences in comparison with aCD patients and HC. In detail, compared to HC, pCD patients displayed higher levels of propionic, butyric, valeric, 2-ethylhexanoic, tetradecanoic, hexadecanoic, and octadecanoic acids. Instead, aCD patients showed increased levels of propionic, isohexanoic, and 2-ethylhexanoic acids, and a lower abundance of isovaleric and 2-methylbutyric acids when compared to HC. In addition, compared to aCD

patients, pCD patients showed a higher abundance of isobutyric and octadecanoic acid. Finally, the immunological analysis of duodenal biopsy revealed a lower percentage of CD4<sup>+</sup> T lymphocytes in pCD infiltrate compared to that observed in aCD patients. The functional characterization of T cells documented a pro-inflammatory immune response in both aCD and pCD patients, but the pCD patients showed a higher percentage of Th0/Th17 and a lower percentage of Th1/Th17.

**Conclusion:** The results of the present study show, for the first time, that the duodenal microbiota of patients with pCD does not differ substantially from that of aCD; however, serum FFAs and local T cells displayed a distinctive profile between pCD, aCD, and HC. In conclusion, our result may help to shed new light on the “gut microbiota-immunity axis,” lipid metabolites, and duodenal immune response in overt CD and pCD patients, opening new paradigms in understanding the pathogenesis behind CD progression.

#### KEYWORDS

potential celiac disease, celiac disease, microbiota, immune response, fatty acids, T cells, cytokines

## Introduction

Celiac disease (CD) is an immune-mediated systemic disorder elicited, in genetically susceptible individuals, by the ingestion of gluten and related prolamines present in barley and rye. CD is a common inflammatory disease that may affect not only the small intestine but also many extra-intestinal sites, with an estimated prevalence of about 1% in the European and North American populations (Karell et al., 2003; Ciccocioppo et al., 2005). Gluten acts in concert with the HLA-DQ2 or -DQ8 genes of predisposition, the mechanistically involved autoantigen tissue transglutaminase (TG2), and the activation of adaptive immune response (CD4<sup>+</sup> T and B cells) leading to enterocyte damage and the development of villous atrophy, the histological hallmark of CD. In particular, in CD patients, incompletely digested gliadin peptides translocate, largely via trans-epithelial transport, from the intestinal lumen to the *lamina propria*, where they stimulate the release of TG2. Once secreted, the enzyme catalyzes the deamidation of specific glutamine in the gliadin peptides into glutamic acid, thus creating epitopes with higher affinity for the molecules DQ2/DQ8 of major histocompatibility complex class II (MHCII). Deamidated gluten peptides bind to HLA-DQ2 or -DQ8 expressed on antigen-presenting cells (APCs) and induce the activation of CD4<sup>+</sup> T cells, which are responsible for intraepithelial lymphocyte (IEL) infiltration, crypt hyperplasia, and villous atrophy, and the subsequent production of anti-endomysial (EMA), anti-tissue transglutaminase (tTG2), and anti-gliadin (AGA) antibodies (Jabri and Sollid, 2009; Monteleone et al., 2010; Husby et al., 2012; Rubio-Tapia et al., 2012; van Bergen

et al., 2015). Gluten-specific CD4 + T cells have also been involved as the primary driver of acute cytokine release and the onset of digestive symptoms after gluten ingestion. In addition, increasing evidence indicates that the induction of a gluten-specific adaptive CD4<sup>+</sup> T-cell response must be preceded by the activation of the innate immune system and that mast cells, key players of the innate immune response, contribute to the pathogenesis of CD [as reviewed in Anderson (2020) and Frossi et al. (2019)].

The innate and adaptive immune responses, working in synergism, lead to villous atrophy and a variable combination of clinical manifestations, from the classical syndrome of malabsorption to light or even the absent of symptoms (Ludvigsson et al., 2013).

On the other hand, potential CD (pCD) is a condition characterized by the presence of positive CD serology and genetic susceptibility, but a normal (Marsh 0) or almost normal (Marsh 1–2) jejunal mucosa without villous atrophy (Troncone et al., 1996). The expression pCD was first proposed by Ferguson (Ferguson et al., 1993) for patients who do not have and never have had a jejunal biopsy consistent with overt CD and yet have immunological abnormalities similar to those found in CD. In detail, features that are good candidate markers of pCD include the presence of serum endomysial antibodies, a high count of villous intraepithelial lymphocytes, an increased density of IEL expressing  $\gamma\delta$  T-cell receptor, and signs of activated mucosal cell-mediated immunity, such as expression of CD25 and B7 by *lamina propria* mononuclear cells. Moreover, pCD is also characterized by enhanced expression of MHC class II molecules on the

epithelium and on adhesion molecules in the lamina propria, all of which are enhanced by *in vitro* gluten challenge (Maiuri et al., 1996), a coeliac-like intestinal antibody pattern, and a positive rectal gluten challenge (Troncone et al., 1996).

This particular condition is usually considered a clinical challenge because, though pCD represents the early CD stage and its diagnostic criteria are clear, many questions are still unsettled and the results of the studies conducted so far are still conflicting (Kurppa et al., 2009; Tosco et al., 2011; Lionetti et al., 2012; Biagi et al., 2013; Zanini et al., 2013; Auricchio et al., 2014).

In addition to the involvement of the immune system, recent evidence reported several modifications in the intestinal tract microenvironment of CD patients, suggesting a role for gut microbiota in CD onset and persistence (Abdukhakimova et al., 2021). It is known that the gut bacteria play many fundamental roles for the host, such as the synthesis of many nutrients and metabolites (Gibiino et al., 2017; Thursby and Juge, 2017; Valdes et al., 2018), maintenance of the intestinal epithelial integrity (Khosravi and Mazmanian, 2013), and notably, the modulation of immune responses (Brestoff and Artis, 2013; Woo and Alenghat, 2017).

Furthermore, the production of short-chain fatty acids (SCFAs), the major bacterial fermentative end products, reflects the intestinal microbiota composition and activity. In particular, given that SCFAs are crucial to maintaining the host's normal gut physiology and metabolic functions and that a part of them enters the systemic circulation (den Besten et al., 2013), it is tempting to speculate that the gut microbiota could be also related to free fatty acid (FFA) levels. FFAs, classified as short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), and long-chain fatty acids (LCFAs), derive from microbial and host metabolism and, especially MCFAs and LCFAs, are introduced into the diet (i.e., milk and dairy products). Since FFAs modulate the production of chemokines and cytokines (Frommer et al., 2015; Honda et al., 2015; Hung et al., 2015), an altered FFA profile has been associated with the risk of developing a range of disorders in which the immune system is involved, including CD (Aghdassi et al., 2011; Nishi et al., 2014; van Hees et al., 2014; Dai et al., 2015; Rodriguez-Carrion et al., 2016).

Therefore, given that pCD is a valuable biological model of the pathway leading to small intestinal mucosal damage in genetically predisposed subjects, our present study aimed to evaluate whether microbial, lipid, and immunological signatures could better characterize pCD from the overt CD condition. In addition, we also evaluated whether and how immunological peculiarity affects the composition of the duodenal microbiota and its metabolic activity, opening new paradigms in understanding the basic mechanisms involved in the development of small intestinal villous atrophy.

## Materials and methods

### Patient recruitment

Twenty-seven CD patients with positive IgA anti-endomysial (EMA) and anti-tissue transglutaminase (tTGA) antibodies consecutively observed at the Tuscany Regional Referral Center for adult CD were enrolled in this observational study from January 2018 to December 2019. Twelve healthy controls, with negative EMA and tTGA antibodies, and without any other clinical problems, were enrolled among the internal staff participating in the study.

Inclusion criteria were as follows: Age between 18 and 70 years, absence of any form of immunodeficiency, in particular selective IgA deficiency, and patients resident in Tuscany who, in the past 5 years, have not made any trips to countries outside Europe. This last criterion is very relevant for the study of the composition of intestinal microbiota. All patients recruited received adequate information on the study, the objectives, and how they will have to provide the organic samples for analysis. Each patient signed and dated the informed consent.

Exclusion criteria were as follows: Treatment with antibiotics or probiotics during the previous 2 months, acute gastrointestinal infections 1 month before the enrolment, pregnant and breastfeeding women (ongoing or scheduled for the next 48 weeks), the concomitant presence of established malignant neoplasms or chronic inflammatory bowel diseases (Crohn's disease and ulcerative colitis), and patients who have used immunosuppressive drugs in the previous 3 months.

### Patient classification

According to mucosal histology, patients were divided into two groups: aCD (5 men, 12 women, mean age 35.6 years, age range 17–56 years) and pCD (two men, 8 women, mean age 38.8 years, age range 21–50 years). In agreement with the definition of Troncone (Troncone et al., 1996), we decided to include cases with Marsh 2 in the pCD group, given the absence of villous atrophy in these patients. Moreover, 12 healthy controls (five men, seven women, mean age 33.6 years, age range 23–50 years), with negative EMA and tTGA antibodies, served as a control group (HC). For ethical reasons, esophagogastroduodenoscopy with small bowel biopsies was not performed in this group. All the study participants were on a gluten-containing diet and did not consume any drug at the time of examination.

### Sample collection

After an overnight fast, venous blood samples were collected, and three serum aliquots per patient were immediately frozen and stored at  $-80^{\circ}\text{C}$  until further use. All patients underwent an esophagogastroduodenoscopy, and 4–6 small bowel biopsies were taken from the distal part of the duodenum.

Small intestinal mucosal damage was graduated according to the classification of Marsh modified by [Oberhuber et al. \(1999\)](#).

## Antibody testing

The tTGA levels were measured by a commercially available enzyme-linked immunosorbent assay kit (EutTG, Eurospital, Trieste, Italy) that employs human recombinant tTG as antigen. EMA antibodies were determined by indirect immunofluorescence, using tissue sections of monkey esophagus (Eurospital) as previously reported ([Vignoli et al., 2019](#)).

## Generation of t-cell clones from intestinal infiltrating lymphocytes

Duodenal mucosa samples were collected in RPMI 1640 culture medium (EuroClone, Italy) and dissociated with the Tumor Dissociation Kit, human (Miltenyi Biotech, UK) in combination with the gentleMACS™ Octo Dissociator (Miltenyi Biotech, Germany) to isolate tissue-infiltrating lymphocytes (TILs). TILs were magnetically isolated with anti-human CD3<sup>+</sup> microbeads (MiltenyiBiotec, UK) using the AutoMACS Pro Separator (Miltenyi Biotech, Germany) and cloned under limiting dilution. Single T-cell clones (Tcc) were seeded in microwells (0.3 cells/well) containing RPMI 1640 supplemented with 10% FBS HyClone (Hyclone Laboratories, South Logan, Uthan), in the presence of  $2 \times 10^6$  irradiated (9,000 rad) peripheral blood mononuclear cells (PBMCs), phytohemagglutinin (PHA, 0.5% vol/vol; EuroClone, Italy), and recombinant human interleukin-2 (IL-2, 50 U/ml; Eurocetus, Italy). At weekly intervals,  $2 \times 10^6$  irradiated PBMCs and IL-2 were added to each micro-culture to maintain the expansion of growing clones.

## Phenotypic and functional characterization of isolated T-cell clones

The expression of Tcc surface markers (CD4 and CD8) was analyzed by flow cytometry using a BD FACSCanto™ II, and a total of  $10^4$  events for each sample was acquired. To assess cytokine profile, Tcc markers were resuspended at a concentration of  $10^6$  cells/ml medium and cultured for 48 h in the presence of PHA (1%). Cell-free supernatants were collected and assayed in duplicate for IFN- $\gamma$ , IL-4, IL-17, and IL-10 content by commercial ELISA kits (BioLegend, San Diego). The supernatants presenting cytokine levels that were 5 SD above the mean levels of control supernatants derived from irradiated antigen-presenting cells alone were regarded as positive. Based

on the cytokine profile and the CD4/CD8 expression, we divided the Tcc into the following groups: Th1-Tc1 (only IFN- $\gamma$ ), Th2-Tc2 (only IL-4), Th17-Tc17 (only IL-17), Treg-Tcreg (only IL-10), Th0-Tc0 (IL-4 + IFN- $\gamma$ ), Th1/Th17-Tc1/Tc17 (IFN- $\gamma$  + IL-17), and Th0/Th17-Tc0/Tc17 (IL-4 + IFN- $\gamma$  + IL-17).

## Deoxyribonucleic acid extraction and bioinformatics analysis of 16S ribosomal ribonucleic acid

DNA extraction and bioinformatics analysis of 16S rRNA were performed as previously described ([Niccolai et al., 2020](#)). Briefly, total DNA was extracted from duodenal mucosa samples of aCD and pCD patients using the DNeasyPowerLyzerPowerSoil Kit (Qiagen, Hilden, Germany) from frozen samples (-80°C) according to the manufacturer's instructions. The quality and quantity of extracted DNA were assessed using the Qubit Fluorometer (Thermo Fisher Scientific) and then genomic DNA was frozen at -20°C.

Extracted DNA samples were sent to NEXT Genomics (Sesto Fiorentino, Italy) where amplicons of the variable V3–V4 region of the bacterial 16s rRNA gene were sequenced in paired-end mode ( $2 \times 300$  cycles) on the Illumina MiSeq platform, according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol ([Pagliai et al., 2020](#); [Russo et al., 2021](#)). Raw sequences were processed following the software pipeline MICCA ([Albanese et al., 2015](#)). Paired-end reads were assembled using the “mergepairs” command, maintaining a minimum overlap of 100 bp and an edit distance in the maximum overlap of 32 bp. Subsequently, the sequences were cut with the “trim” command in order to remove the primers and eventually eliminate the reads with imperfections in primer sequences. All the reads with a length lower than 350 bp and with an error rate higher than or equal to 0.5 were removed with the “filter” command. Cleaned reads were eventually merged into a single file with the “merge” command and transformed into a fasta file. The OTUs were generated using the “otu” command in “denovo\_greedy” mode, setting a 97% identity and performing an automatic removal of chimeras with the “-c” option. The longest sequence of each OTU was used for the taxonomic assignment using the “classify” command in “rdp” mode, i.e., using the RDP Bayesian classifier (10.1093/nar/gki038) that is able to obtain classification and confidence for taxonomic ranks up to genus level.

## Microbial community analysis

Statistical analyses on the bacterial community were performed in R ([R Development Core Team, 2014](#)) with the help of the packages phyloseq 1.26.1 ([McMurdie and Holmes, 2013](#)), DESeq2 1.22.2 ([Love et al., 2014](#)), and other packages

satisfying their dependencies, in particular *vegan* 2.5–5 (Willis and Bunge, 2015). Rarefaction analysis on OTUs was performed using the function *rarecurve* (step 50 reads), further processed to highlight saturated samples (arbitrarily defined as saturated samples with a final slope in the rarefaction curve with an increment in OTU number per reads  $< 1e-2$ ). For the cluster analysis (complete clustering on Euclidean distance) of the entire community, the OTU table was first normalized using the total OTU counts of each sample and then adjusted using square root transformation. The coverage was calculated by Good's estimator using the formula:  $(1 - n/N) \times 100$ , where  $n$  is the number of sequences found once in a sample (singletons), and  $N$  is the total number of sequences in that sample.

Richness, Shannon, Chao 1, and evenness indices were used to characterize the ecological properties of each sample using the function *estimate\_richness* from *phyloseq* (McMurdie and Holmes, 2013). The evenness index was calculated using the formula  $E = S/\log(R)$ , where  $S$  is the Shannon diversity index and  $R$  is the number of OTUs in the sample. Differences in all indices between overt CD and pCD were tested using a paired Wilcoxon signed-rank test. The differential analysis of

abundance at the OTUs, as well as at the different taxonomic ranks (created using the *tax\_glom* function in *phyloseq*), was performed with *DESeq2* (Love et al., 2014).

## Evaluation of free fatty acids by gas chromatography-mass spectrometry analysis

The analysis of FFAs was performed by Agilent gas chromatography-mass spectrometry (GC-MS) system composed of 5971 single quadrupole mass spectrometer, 5890 gas-chromatograph, and 7673 autosampler, with a dedicated previously described protocol (Baldi et al., 2021). The chemicals, GC-MS conditions, and calibration parameters were reported in supporting information SX. Just before the analysis, each sample was thawed. The FFAs were extracted as follows: an aliquot of 300  $\mu$ l of serum sample was added to 10  $\mu$ l of ISTD mixture, 100  $\mu$ l of tert-butyl methyl ether, and 20  $\mu$ l of 6 M HCl + 0.5 M NaCl solution in 0.5 ml centrifuge tube. Afterward, each tube was stirred in a vortex for 2 min,

TABLE 1 Patients' clinical parameters.

Patients	Codes	Age	Sex	Anti-tTG IgA (U/ml)	Anti-EMA-IgA	Diagnosis	Histology (grade marsh)
Patient 1	IMM2	18	M	24.1	Positive	aCD	3C
Patient 2	IMM5	21	M	22.2	Positive	aCD	3B
Patient 3	IMM6	24	F	97.8	Positive	aCD	3A
Patient 4	IMM8	28	F	28.0	Positive	aCD	3A
Patient 5	IMM9	54	F	14.4	Weakly positive	pCD	0
Patient 6	IMM10	21	F	13.8	Positive	pCD	1
Patient 7	IMM11	19	F	23.4	Positive	aCD	3A
Patient 8	IMM13	29	F	30.9	Positive	pCD	2
Patient 9	IMM22	31	F	>100	Positive	pCD	0
Patient 10	IMM26	28	F	>100	Positive	aCD	3A
Patient 11	IMM27	52	F	>100	Positive	aCD + HD	3A
Patient 12	IMM31	45	F	>100	Positive	aCD	3B
Patient 13	IMM35	41	F	95.3	Positive	aCD	3A
Patient 14	IMM39	48	M	>100	Positive	pCD + DH	0
Patient 15	IMM40	54	F	>100	Positive	aCD	3B
Patient 16	IMM41	19	F	12.2	Positive	aCD	3A
Patient 17	IMM48	17	M	>100	Positive	aCD	3C
Patient 18	IMM51	40	M	17.0	Positive	aCD	3B
Patient 19	IMM59	41	F	19.7	Positive	pCD + HD	2
Patient 20	IMM60	43	F	30.4	Positive	pCD	1
Patient 21	IMM61	30	F	11.2	Weakly positive	pCD	1
Patient 22	IMM68	41	F	47.0	Positive	aCD	3A
Patient 23	IMM70	41	F	19.1	Weakly positive	pCD	1
Patient 24	IMM73	46	F	92.0	Positive	aCD	3B
Patient 25	IMM82	56	M	32.0	Positive	aCD + HD	3A
Patient 26	IMM108	50	M	13.5	Weakly positive	pCD	1
Patient 27	IMM109	56	M	>100	Positive	aCD	3B

DH, herpetiform dermatitis.

centrifuged at 10,000 rpm for 5 min, and finally, the solvent layer was transferred to a vial with a microvolume insert and analyzed.

## Statistical analysis

The statistical analysis of immunological and FFAs data was performed using GraphPad Prism (v. 5) software, and the data were expressed as the mean  $\pm$  standard deviation (SD). The comparisons between aCD, pCD patients, and HC were evaluated with the Kruskal–Wallis test, while the comparisons between the Tcc groups were evaluated with the two-tailed Chi-square test or Fisher's exact test, when appropriate (in detail, when  $\leq 20\%$  of cell counts were less than 5, we used the Chi-square test; if  $> 20\%$  of expected cell counts were less than 5, we used Fisher's exact test). *P*-values  $< 0.05$  were considered statistically significant.

## Results

### Characteristics of the study population

Patients were divided into two groups: aCD (5 men, 12 women, mean age 35.6 years, age range 17–56 years) and pCD (2 men, 8 women, mean age 38.8 years, age range 21–50 years), in accordance with mucosal histology. In more detail, nine aCD patients had partial villous atrophy (Marsh 3 A), six subtotal villous atrophy (Marsh 3B), and two total villous atrophy (Marsh 3C); among pCD patients, histological examination revealed an apparently normal mucosa (Marsh 0) in three subjects, just an increase in intraepithelial lymphocytes (Marsh 1) in five, and increased intraepithelial lymphocytes coupled with crypt hyperplasia (Marsh 2) in two patients (Table 1). Twelve healthy controls (5 men, 7 women, mean age 33.6 years, age range 23–50 years), with negative EMA and tTGA antibodies, served as a control group (HC).

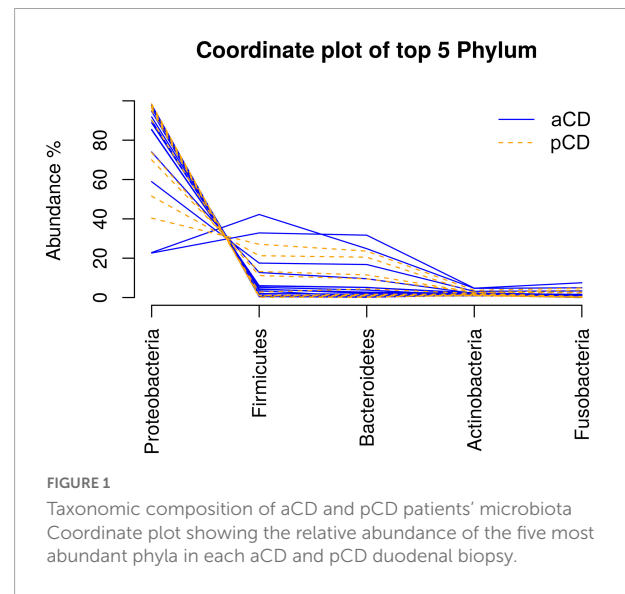
### Analysis of microbiota composition and free fatty acids of atrophic CD and potential celiac disease groups

#### Comparison of tissue microbiota composition between atrophic CD and potential celiac disease patients

We first analyzed the microbiota composition of duodenal mucosa samples obtained for both CD and pCD patients. We obtained a total of 737,729 reads, and after all preprocessing steps (pair merging, trimming, quality filtering, and chimeras removal), a total of 523,825 reads were available for further analysis (Table 2, see Supplementary material for details).

TABLE 2 Summary of the taxonomic analysis of the obtained OTUs.

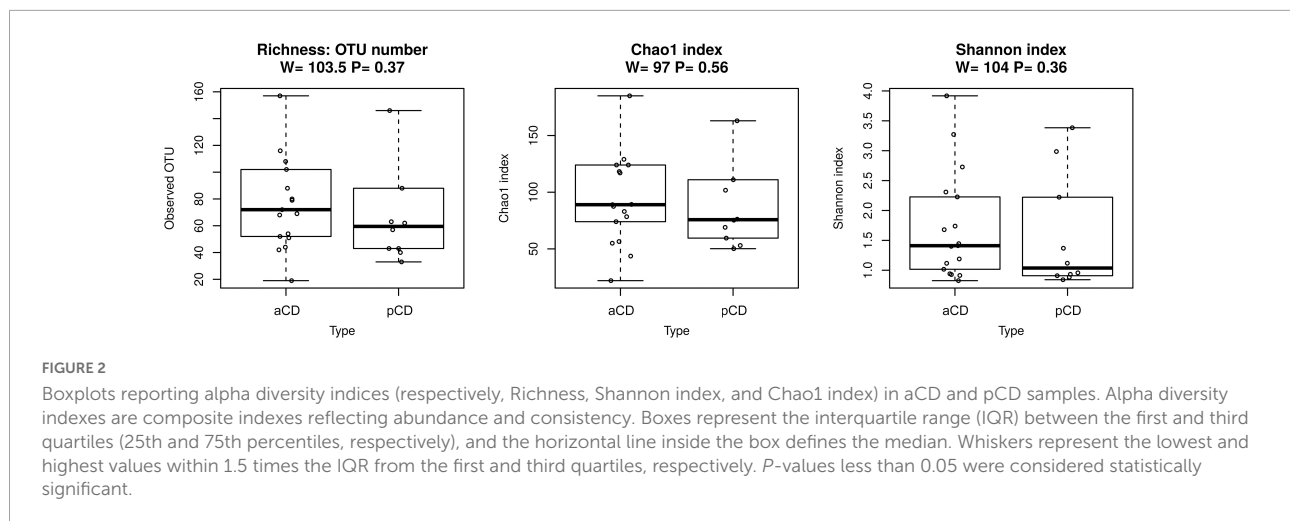
Rank	Counts	Reads	% Reads	OTU	% OTU
Phylum	15	515,816	99.97771	320	99.37888
Class	24	515,570	99.93003	315	97.82609
Order	37	515,570	99.93003	315	97.82609
Family	69	515,264	99.87072	303	94.09938
Genus	116	508,646	98.58799	256	79.50311



As reported in Figure 1, the analysis of the taxonomic composition revealed that more than 99% of the sequences collected could be ascribed to the five most representative phyla: *Proteobacteria* (72.98%), *Firmicutes* (11.92%), *Bacteroidetes* (10.36%), *Actinobacteria* (2.33%), and *Fusobacteria* (2.16%). As shown in Figure 2, no significant difference was reported for richness and Shannon and Chao1 alpha diversity indices. Moreover, in order to investigate the similarity of patients in terms of taxonomy abundance profiles, we evaluated PCoA and NMDS and completed the hierarchical clustering using several data transformations and the Bray-Curtis dissimilarity as a distance metric. Surprisingly and despite our efforts, no evident bacterial groupings were observable between aCD and pCD patients. As evidence of our fine analysis, in Figure 3, we reported several multivariate plots built upon square root transformed percent abundances, namely, a) hierarchical clustering of Euclidean distance, b) hierarchical clustering of the top five most abundant taxa only, and c) a PCoA of Bray-Curtis distance.

#### Evaluation of serum free fatty acids profiles

We evaluated the metabolic profile of the patients performing the qualitative and quantitative analysis of serum FFAs, namely, linear SCFAs (acetic, propionic, butyric, and



valeric acids), branched SCFAs (isobutyric, isovaleric, 2-ethylhexanoic, 2-methylbutyric, and cyclohexanoic acids), ethylhexanoic and cyclohexanoic are MCFAs and not branched SCFAs (hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids), and LCFAs (tetradecanoic, hexadecanoic, and octadecanoic acids), between aCD patients, pCD patients, and HC (Figure 4).

In detail, comparing aCD and pCD patients, the latter showed significantly higher levels of isobutyric ( $p < 0.01$ ) and octadecanoic acids ( $p < 0.01$ ). Moreover, pCD vs aCD patients displayed a higher abundance of propionic ( $p < 0.01$ ), butyric ( $p < 0.01$ ), valeric ( $p < 0.01$ ), 2-ethylhexanoic ( $p < 0.01$ ), tetradecanoic ( $p < 0.05$ ), hexadecanoic ( $p < 0.001$ ), and octadecanoic ( $p < 0.01$ ) acids compared to HC. Finally, aCD patients, in comparison with the healthy controls, showed increased levels of propionic ( $p < 0.05$ ) and 2-ethylhexanoic ( $p < 0.001$ ) acids and a lower abundance of isovaleric ( $p < 0.05$ ) and 2-methylbutyric ( $p < 0.01$ ) acids (Table 3).

## Characterization of mucosal infiltrating T cells in atrophic CD and potential celiac disease patients

We evaluated differences in the T-cell infiltration using biopsies of the duodenal mucosa of aCD and pCD patients; for ethical reasons, we were not able to obtain duodenal biopsies from HC to be used to compare the results.

We cloned and expanded *in vivo*-activated TILs, obtaining a total number of 267 T-cell clones: 206 Tcc from aCD patients and 61 Tcc from pCD patients.

The evaluation of the surface markers showed a prevalence of CD4<sup>+</sup>Tcc in both patient subgroups with a lower percentage in pCD patients [pCD vs aCD: 73.8% (45/61) vs 80.6% (166/206)]. Moreover, the analysis showed 26.2% (16/61) CD8<sup>+</sup>Tcc in the duodenal mucosa of pCD patients vs 17%

(35/206) CD8<sup>+</sup>Tcc in the duodenal mucosa of aCD patients, 0.5% (1/206) CD4<sup>+</sup>/CD8<sup>+</sup>Tcc, and an interesting percentage (1.9%, 4/206) of CD4<sup>-</sup>/CD8<sup>-</sup>Tcc only in the gut mucosa of aCD patients (Figure 5).

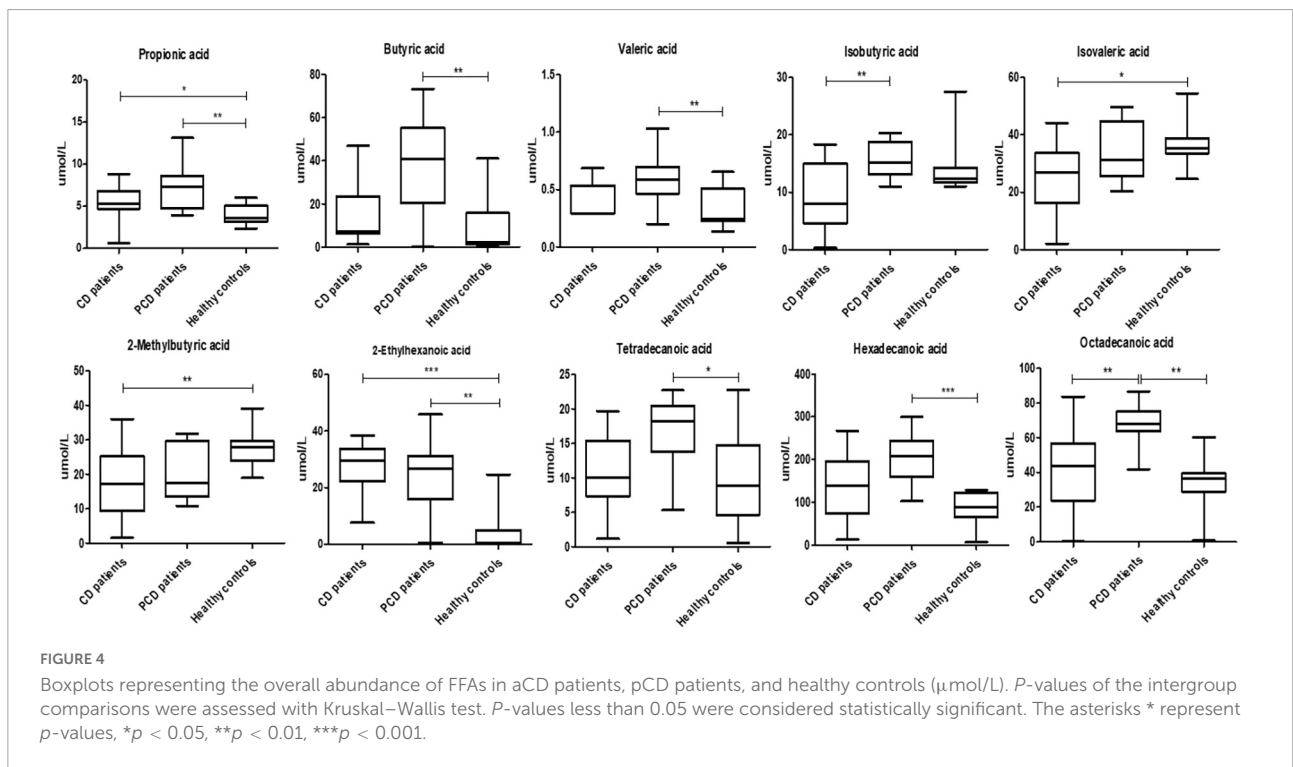
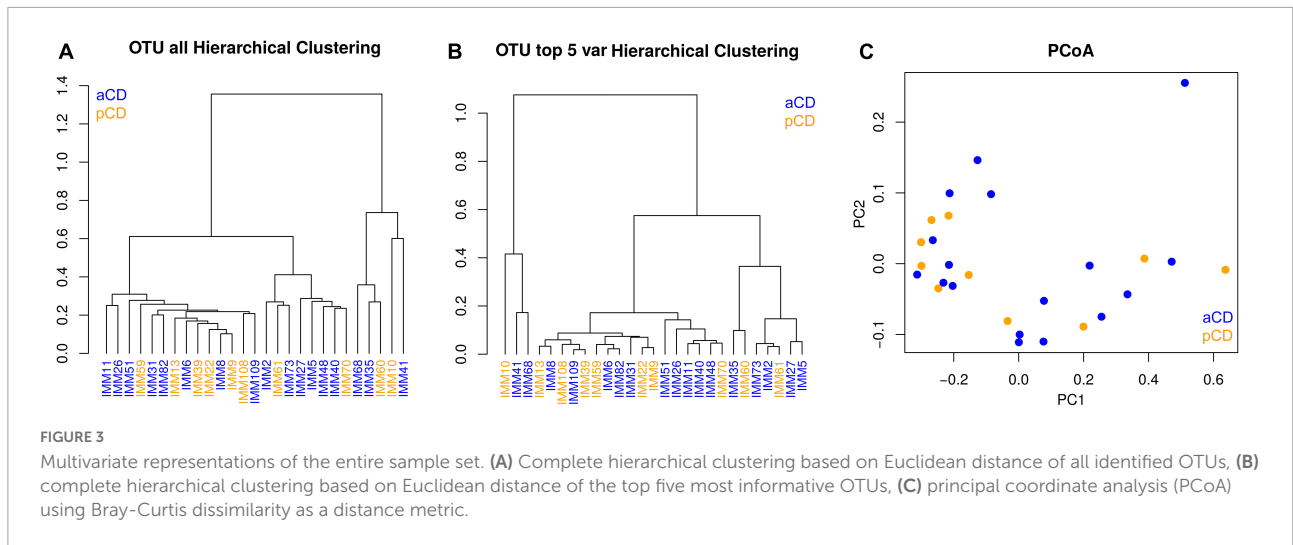
Based on the cytokine profile of CD4<sup>+</sup> Tcc, most of them produced IFN- $\gamma$  [pCD vs aCD: 86.6% (39/45) vs 80.7% (134/166)] (Two-tailed Chi-square test,  $p = 0.3574$ ). In addition, the fine analysis of the CD4<sup>+</sup> T-cell subsets (Figure 6A) revealed that the duodenal mucosa of pCD patients showed lower percentages of Th1/Th17 [pCD vs aCD: 11.1% (5/45) vs 36.7% (61/166)] ( $p = 0.001$ ), Th0 [pCD: 13.3% (6/45) vs 14.5% (24/166)] ( $p = 0.8481$ ), Th2 [pCD vs aCD: 9% (4/45) vs 16.9% (28/166)] ( $p = 0.2437$ ), and higher percentages of Th1 [pCD vs aCD: 33.3% (15/45) vs 24.7% (41/166)] ( $p = 0.2446$ ), Th0/Th17 [pCD vs aCD: 28.9% (13/45) vs 4.8% (8/166)] (Two-tailed Chi-square test,  $p < 0.0001$ ), Th17 [pCD vs aCD: 2.2% (1/45) vs 1.2% (2/166)] ( $p = 0.5149$ ), and Treg [pCD vs aCD: 2.2% (1/45) vs 1.2% (2/166)] ( $p = 0.5149$ ).

Regarding the cytokine profile of CD8<sup>+</sup> population, we documented again that the majority produced IFN- $\gamma$  [pCD vs aCD: 93.75% (15/16) vs 97.1% (34/35)] ( $p = 0.5333$ ).

In addition, the analysis of CD8<sup>+</sup> T-cell subsets (Figure 6B) showed in the duodenal mucosa of pCD patients lower percentages of Tc1/Tc17 [pCD vs aCD: 12.5% (2/16) vs 20% (7/35)] ( $p = 0.7012$ ), Tc0 [pCD vs aCD: 0% vs 5.7% (2/35)] ( $p = 1.0000$ ), Tc2 [pCD vs aCD: 0% vs 2.9% (1/35)] ( $p = 1.0000$ ), and higher percentages of Tc1 [pCD vs aCD: 75% (12/16) vs 71.4% (25/35)] ( $p = 0.7909$ ), Tc0/Tc17 [pCD vs aCD: 6.25% (1/16) vs 0%] ( $p = 0.3137$ ), and Tc17 [pCD vs aCD: 6.25% (1/16) vs 0%] ( $p = 0.3137$ ).

## Discussion

In this original study, we show for the first time that the duodenal microbiota of patients with pCD does not differ



substantially from that of aCD. On the other hand, we observed differences in the local immune T-cell population (increased Th0/Th17 and reduced Th1/Th17 in pCD), while serum FFAs displayed a distinctive profile between pCD, aCD, and HC.

Although great advances have been made in understanding the role of CD adaptive immune mechanisms in response to gluten peptides, and their crosstalk with the intestinal microbiota, the complete set of pathogenic events causing the development of tissue lesions remains unclear. In our study, therefore, we explored for the first time the complex crosstalk between the microbiota, lipid metabolites, and the T-cell profile

in both aCD and pCD patients, to better characterize aspects still unclear in the pCD condition and assess further significant differences between the two faces of celiac disease.

First, we compared the mucosal microbiota composition in the duodenal biopsies of aCD and pCD patients. Since most studies have been conducted using both different sequencing methods and inhomogeneous sampling, the findings on the gut microbiota composition of aCD patients are highly heterogeneous and sometimes contrasting.

Previous studies (Nistal et al., 2012; Cheng et al., 2013) showed that the most abundant bacterial phyla in CD adults



TABLE 3 SCFA, MCFA, and LCFA abundance in aCD and pCD patients and HC.

SCFAs ( $\mu$ mol/L)	aCD	pCD	HC
Acetic acid	119.21 $\pm$ 57.46	121.91 $\pm$ 26.32	127.17 $\pm$ 48.38
Propionic acid	5.07 $\pm$ 2.04	7.37 $\pm$ 2.82	4.10 $\pm$ 1.19
Butyric acid	5.93 $\pm$ 5.46	37.61 $\pm$ 23.04	11.50 $\pm$ 16.18
Isobutyric acid	6.06 $\pm$ 4.18	15.73 $\pm$ 3.30	12.36 $\pm$ 1.15
2-Methylbutyric acid	13.85 $\pm$ 6.58	20.26 $\pm$ 7.98	26.44 $\pm$ 4.16
Isovaleric acid	19.88 $\pm$ 9.32	33.28 $\pm$ 9.93	35.10 $\pm$ 4.99
Valeric acid	0.33 $\pm$ 0.12	0.58 $\pm$ 0.22	0.35 $\pm$ 0.18
<b>MCFAs (<math>\mu</math> mol/L)</b>			
Hexanoic acid	1.79 $\pm$ 0.92	2.16 $\pm$ 1.02	2.95 $\pm$ 1.27
Isohexanoic acid	0.09 $\pm$ 0.03	0.09 $\pm$ 0.03	0.06 $\pm$ 0.01
2-Ethylhexanoic acid	29.77 $\pm$ 4.36	24.21 $\pm$ 13.87	5.31 $\pm$ 9.27
Cyclohexanoic acid	0.29 $\pm$ 0.13	0.43 $\pm$ 0.06	0.31 $\pm$ 0.01
Heptanoic acid	0.35 $\pm$ 0.29	0.08 $\pm$ 0.03	0.09 $\pm$ 0.08
Octanoic acid	1.43 $\pm$ 0.33	1.43 $\pm$ 0.33	1.18 $\pm$ 1.57
Non-anoic acid	0.06 $\pm$ 0.03	0.06 $\pm$ 0.03	0.05 $\pm$ 0.01
Decanoic acid	2.42 $\pm$ 0.92	2.42 $\pm$ 0.92	2.45 $\pm$ 2.60
Dodecanoic acid	3.45 $\pm$ 1.75	3.45 $\pm$ 1.75	2.19 $\pm$ 2.48
<b>LCFAs (<math>\mu</math> mol/L)</b>			
Tetradecanoic acid	16.61 $\pm$ 5.30	16.61 $\pm$ 5.30	8.72 $\pm$ 7.27
Hexadecanoic acid	204.93 $\pm$ 61.25	204.93 $\pm$ 61.25	76.13 $\pm$ 41.66
Octadecanoic acid	68.40 $\pm$ 12.77	68.40 $\pm$ 12.77	30.84 $\pm$ 16.85

Data are presented as median (standard deviation). aCD, atrophic celiac disease; pCD, potential celiac disease; HC, healthy control; SCFAs, short-chain fatty acids; MCFAs, medium-chain fatty acids; LCFAs, long-chain fatty acids.

were *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, whereas CD children displayed higher abundances of *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. In accordance with these findings, we observed an abundance of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* in both CD and pCD patients; however, in contrast with the previous results, we observed high percentages of *Proteobacteria* (72.98%) and *Fusobacteria* in adult CD and pCD patients.

The high abundance of Gram-negative *Proteobacteria* may result in altered exposure of intestinal epithelial cells to bacterial lipopolysaccharides (LPS) and some metabolites, such as SCFAs (Akobeng et al., 2020). Intriguingly, no statistically different microbial taxa or bacterial groupings were displayed between the two patients' cohorts, suggesting the existence of a similar microbial consortium in both conditions.

Even though we demonstrated the presence of a stable microbiota composition between overt CD and pCD patients, based on our previous metabolic results (Bertini et al., 2009; Bernini et al., 2011), we hypothesized that the functional metabolic activity of gut microbiota could be differently regulated between the two pathologic conditions. Indeed, in one of our previous studies, by employing a non-invasive metabolomic approach, we have first shown that CD has a well-defined metabolomic signature (Bertini et al., 2009). Notably, sera of CD patients were characterized by lower levels of

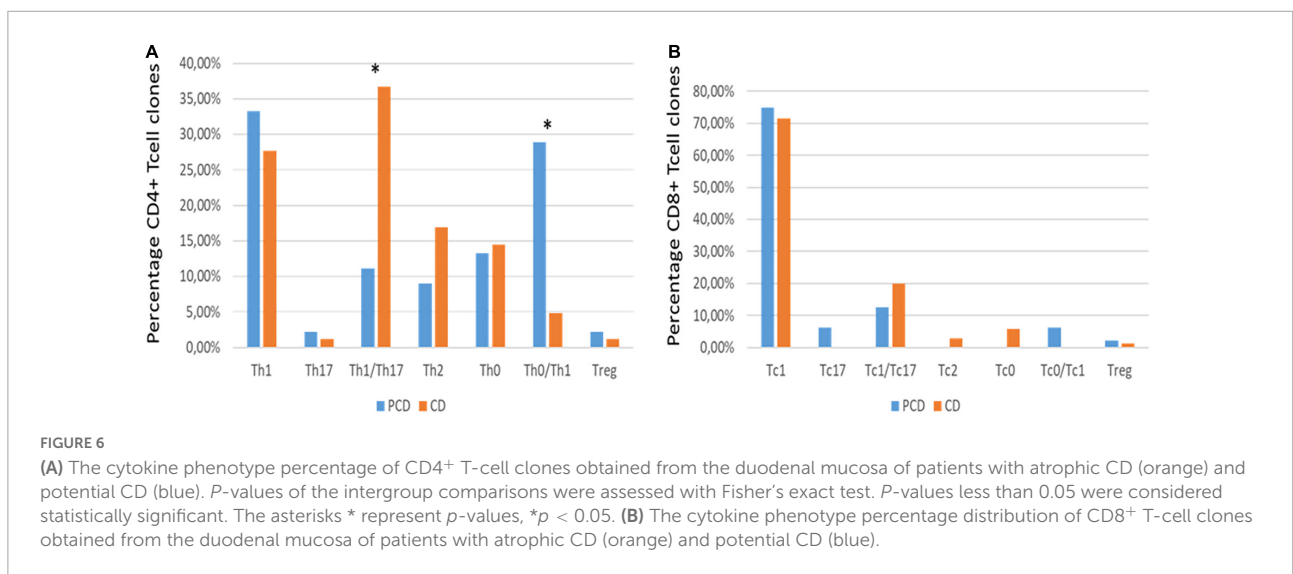
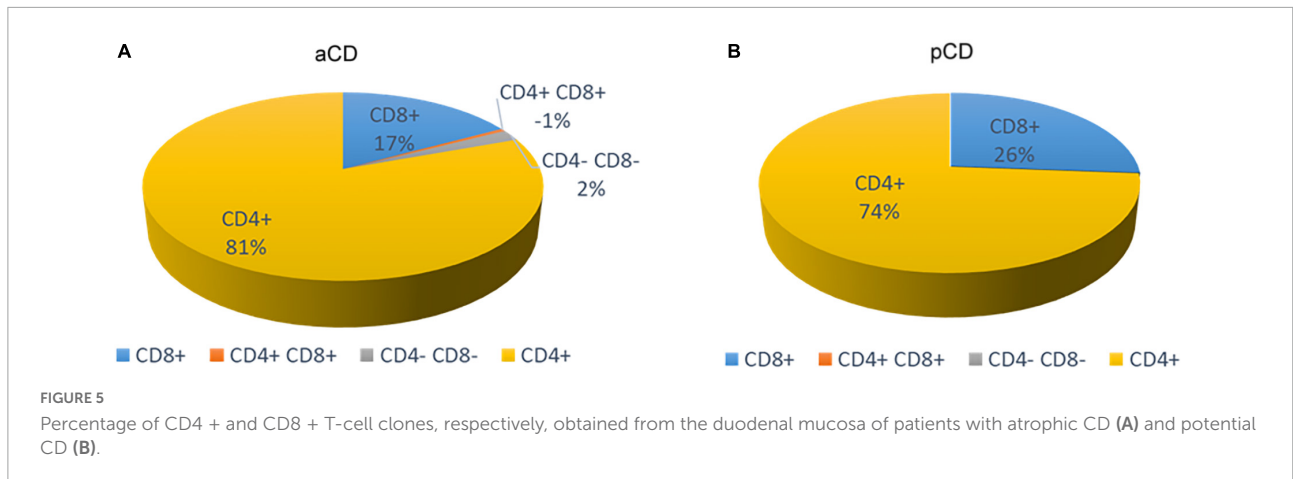
several metabolites, such as amino acids, lipids, pyruvate, and choline, and by higher levels of glucose and 3-hydroxybutyric acid, while urine samples showed altered levels of, among others, indoxyl sulfate, meta-[hydroxyphenyl]propionic acid, and phenylacetyl glycine. Moreover, in another study addressed to pCD and CD patients as well as HC, we observed, examining serum and urine samples, that pCD largely shares the metabolomic signature of aCD (Bernini et al., 2011). Most metabolites found to be significantly different between control and CD subjects were also altered in pCD (Bernini et al., 2011).

Regarding the free fatty acids, altered circulating levels of SCFAs, MCFAs, and LCFAs and LCFAs, have been related to CD (Niccolai et al., 2017; Baldi et al., 2021). In our previous study, we compared the serum FFA profile of patients with CD, adenomatous polyposis (AP), and colorectal cancer (CRC) to HC (Baldi et al., 2021). We observed that HC showed a different composition of FFAs as compared to CRC, AP, and CD patients. Moreover, we demonstrated a strong positive association between CD and butyric acid, suggesting for the first time that it could represent a potential biomarker for CD screening. However, no information addressing the serum SCFA composition of pCD patients is currently available. So, as they provide information on the general health status of both the host and its intestinal microbial ecosystem, we evaluated the abundance of each circulating FFA. In detail, compared to HC, pCD patients displayed higher levels of propionic, butyric, valeric, 2-ethylhexanoic, tetradecanoic, hexadecanoic, and octadecanoic acids. Propionic, butyric, and valeric acids are the most common microbiota-produced SCFAs that, as well established, exert positive metabolic effects on enterocytes, act as tumor suppressor agents, and regulate host gene expression via histone deacetylase inhibition (HDAC; Nankova et al., 2014; Yuille et al., 2018).

Moreover and notably, butyric acid is involved in Tregs differentiation (Kanauchi et al., 1999; Atarashi et al., 2013) and in the maintenance of the gastrointestinal epithelial barrier integrity (Braniste et al., 2014; Buscarinu et al., 2017; Mizuno et al., 2017), thus preventing microbial translocation and LPS-driven triggering of TLR4-mediated signaling.

In contrast to SCFAs, MCFAs and LCFAs arise mostly from dietary triglycerides and are important regulators of energy metabolism, gene expression, and immune processes (Schonfeld and Wojtczak, 2016; Pinkosky et al., 2020). In particular, they can antagonize the anti-inflammatory activities of SCFAs, as they support the Th1 and Th17 differentiation, so both MCFAs and LCFAs could be involved in the onset and persistence of duodenal inflammation in pCD patients, compared to HC.

In aCD patients, the results of our study showed increased levels of propionic, isohexanoic, and 2-ethylhexanoic acids, and a lower abundance of isovaleric and 2-methylbutyric acids as compared to HC. In addition, we found that, compared to aCD patients, pCD patients showed higher abundances of isobutyric and octadecanoic acid. Isobutyric acid is a branched SCFA produced by valine and leucine



fermentation, and a high abundance of this acid indicates an increased amino acid fermentation. So, this finding suggests that, even if the composition of the duodenal microbiota is almost similar, aCD and pCD patients displayed a different microbial functional activity, which deserves to be deepened in future studies.

As there is a documented mutual interplay between the microbiome and the immune response, to assess whether and how immunological peculiarity affects the composition of the duodenal microbiota and its metabolic activity, we also evaluated the cellular immune profile in aCD and pCD patients. In detail, the phenotypic characterization of T cells revealed, in agreement with previous studies, a lower percentage of CD4+ T cells in pCD patients compared to CD patients. It has been shown that CD4+ T cells play a key role in the inflammatory response triggered by gluten, by driving and orchestrating the gut inflammation and tissue destruction in human celiac disease (Voisine and Abadie, 2021).

Moreover, in accordance with Vitale et al., pCD patients showed a high percentage of T cells co-producing IL-17,

IL-4, and IFN-γ (Th0/Th17  $p < 0.00005$ , Tc0/Tc17; Vitale et al., 2019). Notably, IL-17 is a pro-inflammatory cytokine, but a number of studies underlined its potential involvement in the prevention of gut mucosa inflammation (Mucida and Salek-Ardakani, 2009; Cukrowska et al., 2017). In addition, as compared to atrophic CD, pCD patients showed lower percentages of T cells co-producing IFN-γ and IL-17 (Th1/Th17  $p < 0.001$ ). Of note, the Th1/Th17 subpopulation promotes damage to the enterocytes leading to the atrophy of the intestinal villi (Cukrowska et al., 2017). We hypothesize that the increase of Th0/Th17 linked with the decrease of Th1/Th17 might contrast the inflammation, contributing to preventing mucosal damage in the pCD patients. However, whether and how this immunological peculiarity can affect the composition of the duodenal microbiota and its metabolic activity remains unknown.

Therefore, although our exploratory study has some limitations, such as a limited number of patients, little information on patients' dietary pattern, or the relatively low level of taxonomic resolution of 16S rRNA gene metagenomics,

we obtained innovative and interesting preliminary data, such as the same duodenal microbial architecture between aCD and pCD patients but differences in the serum FFA's profiles and in the duodenal adaptive immunity. However, we will try to overcome the mentioned limits in a future larger and more detailed investigation with aCD and pCD patients, divided into homogeneous groups considering also age, gender, nutrition, and comorbidity.

Although the precise relationship between host lipid metabolism and gut microbiota remains to be clarified, our findings suggest a possible modification in microbial functionality that needs to be further explored assessing also the contribution of the colonic microbiota, in order to detect potential new biomarkers of human pCD.

## Data availability statement

The data presented in this study are deposited in the NCBI Gene Expression Omnibus repository, accession number: GSE181070 and the analysis script is available at <https://github.com/matteoramazzotti/papers/tree/main/2021celiac>.

## Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee of the University of Florence, Italy (CE: 10443\_oss, 14/02/2017). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## Author contributions

CAS, AA, and RE designed the study. RF, NG, and LG collected the samples. RF, RD, BS, PM, MM, and BG performed the experiments. RF, RE, BS, and NE analyzed the data. CM and RM analyzed microbiota data. RE and RF wrote the manuscript. RE edited the manuscript. AA, RD, and CAS supervised the

manuscript. RE, AA, and CAS provided for funding acquisition. All authors have approved the final draft submitted.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.886008/full#supplementary-material>

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