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Dietary patterns drive loss of fiber-foraging species in the celiac disease patients gut microbiota compared to first-degree relatives

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Abstract

Background Celiac disease is an autoimmune disorder triggered by dietary gluten in genetically predisposed individuals that primarily affects the small intestine. Studies have reported differentially abundant bacterial taxa in the gut microbiota of celiac patients compared with non-celiac controls. However, findings across studies have inconsistencies and no microbial signature of celiac disease has been defined so far.

Results Here, we showed, by comparing celiac patients with their non-celiac 1st-degree relatives, that bacterial communities of related individuals have similar species occurrence and abundance compared with non-relatives, regardless the disease status. We also found in celiac patients a loss of bacterial species associated with fiber degradation, and host metabolic and immune modulation, as ruminiclostridia, ruminococci, *Prevotella*, and *Akkermansia muciniphila* species. We demonstrated that the differential abundance of bacterial species correlates to different dietary patterns observed between the two groups. For instance, *Ruminiclostridium siraeum*, *Ruminococcus bicirculans*, and *Bacteroides plebeious*, recognized as fiber-degraders, appear more abundant in non-celiac 1st-degree relatives, which have a vegetable consumption pattern higher than celiac patients. Pattern of servings per day also suggests a possible link between these species' abundance and daily calorie intake.

Conclusions Overall, we evidenced that a kinship approach could be valuable in unveiling potential celiac disease microbial traits, as well as the significance of dietary factors in shaping microbial profiles and their influence on disease development and progression. Our results pave the way for designing and adopting novel dietary strategies based on gluten-free fiber-enriched ingredients to improve disease management and patients' quality of life.

Keywords Celiac disease, Gut microbiota, Fiber-degraders, *Akkermansia*, Ruminococci bacteria, Gluten-free diet

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Background

Celiac disease (CeD) is an inherited autoimmune disease triggered by ingestion of gluten-containing food in individuals carrying the histocompatibility complex class II HLA-DQ2 and/or HLA-DQ8 haplotypes that primarily affects the small intestinal mucosa [1]. In these patients, dietary gluten, partially digested by gastric, pancreatic, and mammalian small intestinal brush-border membrane enzymes, translocates the intestinal epithelial barrier and activates both an adaptive and innate immune response. The adaptive immune response leads to antibody responses characteristic of CeD, with the production of antibodies against deamidated gliadin peptides and against the self-enzyme tissue transglutaminase 2, making CeD an autoimmune disease. The only one with a known trigger, gene, and autoantibody [2].

Although 40% of the American and European populations have a genetic predisposition to CeD, only 2 to 3% develop the disease [1], indicating that genetic predisposition is necessary but not sufficient for disease development. In addition, in about 20–50% of patients, a gluten-free diet, the only currently available treatment, fail to resolve symptoms. Thus, additional environmental factors, besides dietary gluten, have been proposed to be involved in CeD onset. Recent research indicates that an altered bacterial microbiota might be a contributing factor [3].

Several clinical studies have reported differentially abundant gut bacteria in at-risk individuals or patients with active CeD compared with healthy or non-CeD controls. Most studies report lower abundance of beneficial bacteria, such as *Bifidobacterium* and former *Lactobacillus* species, and an increased abundance of members of the Pseudomonadota (former Proteobacteria) phylum in either duodenal biopsies and stool samples of children or adults with active or remitted CeD compared with healthy or non-CeD groups [4–7]. Moreover, patients with gastrointestinal manifestations had increased prevalence of Pseudomonadota compared with CeD patients with extraintestinal symptoms [8]. Pseudomonadota were also linked to persistent symptoms despite CeD patients following a gluten-free diet [9]. Gram-positive bacteria belonging to Staphylococcaceae family have also been reported in higher prevalence in duodenal biopsies and stool samples of active CeD patients compared with non-CeD control groups [10, 11]. Studies have also shown differences in microbiota compositions in genetically susceptible children who end up developing CeD compared to those who remain healthy [12–14]. Differences among *Bacteroides* species have also been consistently reported, specifically between infants with high genetic risk and standard genetic risk—heterozygous for DQ2 or DQ8 or carrying both DQ2 and DQ8—for CeD [4]. High-risk infants were also characterized by having an unstable microbiota until 24 months old, maturing later in development, and having different gut microbiota composition from those with CeD standard genetic risk [4]. Although these findings provide an important ground to understand alterations in the gut microbiota that can contribute to CeD development, some remain inconsistent across studies and other studies describe no differences between CeD patients and non-CeD/healthy individuals [4, 5].

Minimizing gluten in diet is the primary strategy to tackle intestinal damage and relieving CeD symptomatology [15]. However, even small traces of gluten in diet can re-trigger symptoms and intestinal damage, leaving the gluten-free diet as the most effective and single therapy for patient care management [1]. Grasping how the disease works, specifically its onset, i.e. how a person moves from a status of having a genetic predisposition to developing the CeD specific autoimmunity, irreversibly becoming a CeD patient, and finding alternative treatments to treat them, are crucial to improve these patients' quality of life.

The purpose of our study was to investigate if the gut bacterial community of CeD patients and their non-celiac 1st-degree relatives (NC1R) differ in composition and diversity and potentially establish a bacterial signature associated with CeD that could be used as a prognosis biomarker or a therapeutic target. We hypothesize the clinical perspective adopted here can help shed light on the role of gut microbiota features in disease development, given the context of common environmental (e.g. diet, cohabiting) and genetic factors shaping gut microbiota of cases and controls.

Methods

Subjects and sampling

Participants' recruitment was based on volunteer participation in this study. Inclusion criteria were patients diagnosed with CeD, according to the European Society Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria [16], and thus being under a gluten free diet or being a NC1R of a CeD patient who volunteered to participate in the study. Exclusion criteria included having taken antimicrobials 2 weeks before sample collection and/or probiotic medication in the month before sample collection, pregnancy (in case of women), or being a NC1R of a CeD patient that did not volunteer for the study. Apart from voluntarily offering a stool sample, participants also answered a detailed questionnaire that addressed sociodemographic, diet, medical history, and comorbidities information. The study was approved by the Ethics Committee of Leiria Hospital Center. Informed consent was obtained from all

participants or legal representatives before inclusion in the study. Stool samples were collected at home by study participants and kept at – 20 °C until processing, no longer than 24 h. Upon arrival to the laboratory, samples were immediately aliquoted and cryopreserved at – 80 °C until further analysis.

DNA extraction and *rrn* amplicons

Total DNA was extracted from 100 mg stool samples using the QIAamp Fast DNA Stool Mini kit (Qiagen) according to manufacturer's instructions with slight changes. In brief, 1 ml of InhibitEX buffer was added to each stool sample and the samples were subjected to mechanical lysis by bead-beating using the FastPrep-24™ 5G Instrument (MP Biomedicals) for 30 s at 6.0 m/s before completing the standard protocol. DNA was quantified using a Qubit 4 fluorometer (Thermo Fisher Scientific) combined with the dsDNA high sensitivity (HS) assay kit (Thermo Fisher Scientific). Extracted DNA was kept at—20 °C until use. The *rrn* region (~4500 bp) was amplified by PCR using the NZYProof 2X Green Master Mix (NZYTech). The primers 27F (AGAGTTTGATCMTGGCTCAG) and 2241R (ACCGCCCCAGTHAAACT) were used and barcoded with unique 38-mer sequence at the 5′ end of primers in order to multiplex and distinguish samples in 1 sequencing run and during data analysis. PCR reactions contained 2.0 µl of DNA at a concentration of 1–10 ng/µl. PCR thermal cycling proceed at 95 °C for 3 min and 30 cycles of 95 °C for 30 s, 50 °C for 20 s, and 72 °C for 4 min, with a final extension at 72 °C for 4 min. Dual-barcoded PCR products were purified using the NZYGelpure kit (NZYTech) and quantified with the Qubit 4 fluorometer (Thermo Fisher Scientific) combined with the dsDNA high sensitivity (HS) assay kit (Thermo Fisher Scientific).

Library preparation, sequencing and nanopore data processing

A maximum of 25 samples were combined in equimolar proportions (60 ng per sample) to reach starting material for library prep. In total, 6 different libraries were prepared using the SQK-LSK109 sequencing kit (Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's instructions. Approximately, 750 ng each library was individually loaded into FLO-MIN106D (R9.4.1) flow cells (Oxford Nanopore Technologies, Oxford, UK), and sequencing was carried out in a portable MinION™ MkIC sequencer (Oxford Nanopore Technologies, Oxford, UK), operated with *MINKNOW* v22.12.5 software (Oxford Nanopore Technologies, Oxford, UK). Flow cells were primed according to the manufacturer's instructions, and a 24 h sequencing run was allowed in all cases. Passed fast5 files were processed with the *Guppy* v6.1.5 basecaller with default configuration and resulting fastq files were merged to obtain 1 fastq file per sequencing run. A preliminary QC via *fastqc* v0.11.9 [17] was completed to determine the per-sequence quality and length distributions. The per-sequencing run fastq files were filtered to retain sequences with minimum 3000 nt (~55% of theoretical amplicon size) [18] and maximum 6000 nt in length using *cutadapt* v3.5 [19]. Respective fasta files were obtained using *SeqKit* [20]. Sample de-multiplexing was achieved using the 38-mer-barcode + primer sequence information and an ONT-developed Perl script (https://github.com/nanoporetech/barcoding).

Species-level microbiota evaluation

Reads retained after quality and length filtering were mapped against the MIrROR database [21] containing almost 100 k annotated *rrn* bacteria operons via *minimap* v2.15 aligner with *–x map-ont* configuration. High-quality alignments were retained by setting thresholds to keep sequences with ≥ 90% for sequence identity and ≥ 70% for alignment length, proportional to the target sequence length. A species-counting matrix was then generated for downstream ecological analysis. Alpha diversity comprising the Chao index (randomly rarefied data), Shannon index, Simpson's evenness, Simpson's reciprocal index was computed using *vegan* R package. Community-level beta diversity assessment was conducted on R v4.3.2 using functions implemented in libraries *CoDaSeq*, *vegan*, and *Zcompositions* to deal with sparse and compositional nature of microbiome data. Species counts were filtered to retain those with minimal occurrence of 0.2 and a minimum of 200 reads in sum across all samples (*CoDaSeq::codaSeq.filter*). Next, zero imputation was completed using a Bayesian multiplicative replacement algorithm (*Zcompositions::cmultRepl*), and finally, a centered-log ratio was computed (*CoDaSeq::codaSeq.clr*) to transform compositional data. Aitchison distance (Euclidean distance on compositional data) was calculated using *vegan::vegdist* and interpretative multivariate analysis of the microbial communities was completed by dimensionality reduction based on non-metric multidimensional scaling (NMDS, *vegan::metaMDS*).

Statistical analysis

Shapiro–Wilk test was conducted to evaluate the normality of all data distribution before the statistical evaluation. For non-normally distributed data Wilcoxon Rank Sum test was used to establish meaningful differences between groups. When more than 2 groups were evaluated, the pairwise approach was completed following FDR (false-discovery rate) correction for multiple testing. The permutation-based method *vegan::adonis2* was used

to calculate differential composition in microbial communities linked to the primary observation variable and common covariates recorded for all subjects. Generalized linear mixed-effects model (GLM) (*lme4::glmer*) with disease condition (CeD vs NC1R) and random effects (Sex, Age, BMI, sequencing batch) was used to determine differential abundance for bacterial species. Threshold for selection of differential features was p-adj ≥ 0.1. The association between bacterial species abundance and categorical variables recorded for patients and controls was performed via logistic regression (*stats::glm*), and association with numerical variables was estimated via rank-based Kendall's rho parameter. In both cases, correction for multiple testing was computed following the FDR approach, setting threshold of 0.1 for selection of associations. Chi-square test (*stats::chisq.test*) with Montecarlo p-value simulation was achieved to compare distribution of categorical variables between groups. All plots were obtained by using the *ggplot2* R library.

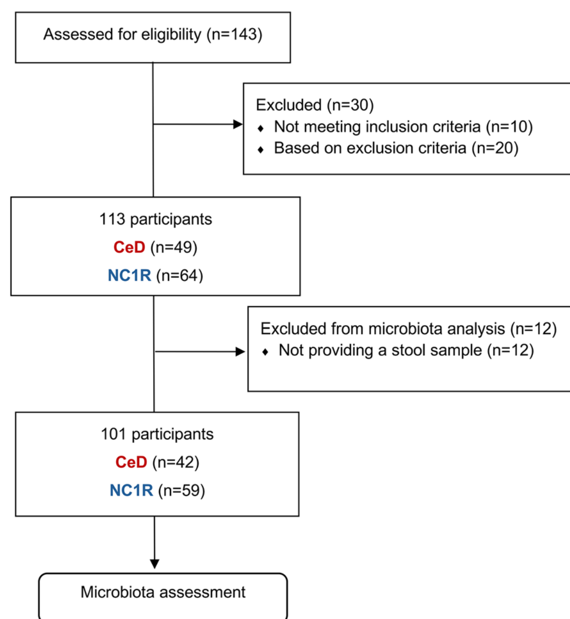

Fig. 1 Study recruiting and exclusion processes. Flow diagram showing the number of participants included: 143 celiac patients (CeD) and their non-celiac 1st-degree relatives (NC1R) volunteered to participate in the current study; out of the 143, 30 were excluded based on inclusion and exclusion study criteria; the final 113 participants were included, but 12 participants failed to provide a stool sample for microbiota assessment

Results

Study population characterization

We recruited a total of 60 CeD patients and 83 of their NC1R. Out of the total 143 volunteer participants, 133 answered the questionnaire that addressed demographic, clinical, dietary and lifestyle factors and 10 were excluded. In addition, 4 CeD patients and 2 NC1R had taken antimicrobials 2 weeks prior to sample collection and 4 CeD patients and 1 non-celiac relative had taken probiotics during the last month. Thus, these volunteers were excluded from the study as well as the 9 NC1R associated to the excluded CeD patients. The recruiting and exclusion processes are represented in Fig. 1.

The study population comprised 40% (n = 49) CeD patients and 60% (n = 64) NC1R (Table 1) (see Supplementary Table 1 for detailed metadata). In both groups, female participants outnumbered male subjects, with a higher ratio in the CeD group (CeD, female/male = 43:6; NC1R, female/male = 36:28), with a statistically significant association between sex and CeD (Chi-test = 13.0, $p < 0.001$). Age distribution ranged from 3 to 58 years old with a mean of 29 ± 14 and a median of 27 years old in the CeD, and 3 to 80 years old in the NC1R with a mean of 40 ± 19 and a median of 45 years old, with a statistically significant difference between the mean ages of both groups ($t = -3.44$, $p < 0.001$). Both groups exhibited diverse education levels, with the majority in each group having either a bachelor's degree or a master's/doctoral degree (CeD, n = 25; NC1R, n = 25). Most of the participants resided in urban areas (CeD, n = 27; NC1R, n = 35) and lived with 3 or 4 people in the same house (CeD, n = 30; NC1R, n = 36).

Age of CeD diagnosis had a wide range, from 1 to 51 years old with a median of 21 years old. Less than a quarter CeD patients included in this study reported having a 1st-degree relative with CeD. Also, in the CeD group, approximately half had another concomitant medical condition (n = 25), with the majority having either 1 or 2 (n = 20) and the remaining 3 comorbidities (n = 5). The most common was a respiratory and/or allergic disease (n = 8), followed by autoimmune thyroiditis (n = 7), anxiety (n = 4), anemia (n = 3), osteopenia (n = 3), and irritable bowel syndrome (n = 3). Within the NC1R, almost half reported having at least 1 medical condition (n = 24). Respiratory and/or allergic disease was also the most common comorbidity in this group (n = 7), followed by hypercholesterolemia (n = 6), hypertension (n = 5), and type 2 diabetes (n = 5). Prevalence of type 2 diabetes and hypertension differed statistically significantly between groups (Chi-test = 8.0, $p = 0.015$), as well as hypercholesterolemia (Chi-test = 6.5, $p = 0.04$).

All individuals in the CeD group followed a gluten-free diet. In the NC1R group almost all had a gluten-containing diet (n = 60). Majority of participants from both groups reported having a regular eating schedule (CeD, n = 40; NC1R, n = 52). Dietary habits indicate most of the individuals in both groups reported eating less than

Table 1 Study population demographic and dietary metadata

Characteristic	CeD (n = 49)	NC1R (n = 64)
Age, years, mean (SD), median, range	29 (14), 27, 3–58	41 (19), 45, 3–80
Female/Male, n	43/6	36/28
Body mass index, kg/m2, mean (SD)	21.0 (2.7)	25.0 (5.0)
Gluten-free diet, n	49	4
Eating schedule		
Irregular, n	9	12
Regular, n	40	52
Number of meals per day		
<3 meals per day, n	1	1
3 meals per day, n	9	29
>3 meals per day, n	39	34
Number of portions per day of grains/grain products		
0 portions, n	0	1
<3 portions, n	26	34
3 portions, n	17	18
>3 portions, n	6	11
Number of portions per day of vegetables		
0 portions, n	1	2
<3 portions, n	34	45
3 portions, n	11	15
>3 portions, n	3	2
Number of portions per day of fruits		
0 portions, n	0	3
<3 portions, n	29	42
3 portions, n	16	12
>3 portions, n	4	7
Number of portions per day of pulses		
0 portions, n	5	5
<3 portions, n	40	49
3 portions, n	4	9
>3 portions, n	0	1
Number of portions per day of dairy		
0 portions, n	5	7
<3 portions, n	31	50
3 portions, n	10	5
>3 portions, n	3	2
Number of portions per day of fish, poultry and eggs		
0 portions, n	1	0
<3 portions, n	31	37
3 portions, n	13	25
>3 portions, n	4	2
Number of portions per day of fats/oils		
0 portions, n	3	3
<3 portions, n	39	51
3 portions, n	6	8
>3 portions, n	1	2
Number of portions per day of sugary drinks and sweets		
0 portions, n	13	16
<3 portions, n	32	43
3 portions, n	4	3
>3 portions, n	0	2
Intake of nutritional supplements in the month prior to sample collection, n	20	12

Table 1 (continued)

CeD celiac disease subjects, *NC1R* non-celiac 1st-degree relatives

3 portions/servings per day of all food groups, including, for instance, grains and grain products (CeD, n = 26; NC1R, n = 34), vegetables (CeD, n = 34; NC1R, n = 45), fruits (CeD, n = 29; NC1R, n = 42), and fish, poultry and eggs (CeD, n = 31; NC1R, n = 37), with no observed significant differences between groups. Interestingly, a statistically significant difference in nutritional supplements intake, like vitamins, minerals, and weight-loss dietary supplements, was found between groups (Chi-test = 6.7, p = 0.012), with a higher intake associated with CeD subjects (CeD, n = 20; NC1R, n = 12). Moreover, only 5 out of the 49 CeD subjects reported being followed by a nutritionist, and of these, only 2 reported intakes of nutritional supplements.

In both groups, the majority reported practicing physical activity (CeD, n = 35; NC1R, n = 34) and non-smoking habits (CeD, n = 46; NC1R, n = 61), while alcohol consumption was limited to less than half of the individuals legally eligible to drink (> 18 years old, according to Portuguese Law) in both groups (CeD, n = 14/38; NC1R, n = 24/52). No significant differences were observed between groups regarding these lifestyle factors.

Microbiota results

Microbiota abundance at the species level was retrieved for 101 samples (CeD, n = 42; NC1R, n = 59). After following strict criteria for filtering out and retaining alignments with high-quality and sequence identity values, we obtained a data matrix with abundance estimation for more than 200 gut microbial species to calculate ecological descriptors with comparative aims between groups. From all descriptors evaluated, individual-based alpha diversity indicates no significant differences between CeD individuals and NC1R, but the last ones tended to have larger number of species present in their samples (adj-GLM = 0.021, Z-value = 1.37, adj-p = 0.170) (Fig. 2A). At the community structure level (beta diversity), we could discern classical host covariates influencing and shaping the gut microbiota. Thus, in addition to the grouping variable (CeD vs NC1R, Adonis = 1.80, R2 = 0.018, p = 0.005) (Fig. 2B), the structure of the gut microbiota was influenced to a similar extent by the sex (Adonis = 1.47, R2 = 0.015, p = 0.036), age (Adonis = 2.26, R2 = 0.022, p < 0.001), and body mass index (BMI) (Adonis = 1.64, R2 = 0.016, p = 0.011), where the disease status and age explained roughly 2% of the observed variability. Consequently, we included such covariation (see methods) in the generalized linear mixed models as random variables, aside from the sequencing batch, to unveil potential microbiota species differentially abundant in CeD individuals.

Before proceeding with the differential abundance approach, we wondered to what extent the kinship perspective adopted in this study could help us distinguish specific microbial signatures for CeD. In such a manner, we compared the Aitchison distance between microbial communities from relatives (patients and their NC1R pairwise comparisons) and non-relatives (remaining pairwise comparisons between non-related individuals) regardless of the disease status. As expected, we found that related individuals had lower Aitchison distance between their gut microbial communities (closer in terms of species occurrence and abundance) (Fig. 2C). Consequently, this cross-sectional framework with a kinship perspective may be crucial for discerning disparate microbial features in similar microbial communities as disease development and progress determinants. Generalized linear mixed models (GLM) with covariate control showed CeD subjects with lower proportions of gut microbiota related to complex polysaccharide and fiber

(See figure on next page.)

Fig. 2 Microbiota assessment on celiac (CeD) subjects and non-celiac 1st-degree relatives (NC1R). Red color legend shows values and distributions for CeD and blue color legend for NC1R (N = 101, CeD = 42, NC1R = 59). **A** Distribution of individual richness alpha diversity descriptor. Statistical comparison based on generalized linear mixed models (GLM, *lme4:glmer* function) with covariate adjustment. **B** Non-metric dimensional scaling (NMDS) analysis of microbiota multivariate data. A permutation-based comparison (Adonis, *vegan::adonis2* function) was used to evaluate the microbial variability attributed to disease condition; statistical estimates are shown in the inbox scatter plot. Ellipses show value distribution and confidence interval at 95%. **C** Aitchison distance (compositional) between relative and non-relative pairs is shown in a boxplot manner. The non-relative distances outnumber several hundred times the obtained for relative pairs. For homogeneous comparison aims, there was a resampling to obtain 250 non-relative random distances to compare with < 100 retrieved from relative pairs. This procedure was tenfold, and statistical comparison was achieved every time, always significantly different (Wilcoxon Rank Sum test). **D** Bacterial species found to be more abundant in non-celiac controls. **E** Species retrieved to be more abundant in CeD subjects. Distribution of clr-based abundance is shown as violin plots; medians appear as solid lines with respective color legends. Statistical comparisons in D and E are based on generalized linear mixed models (GLM, *lme4:glmer* function) with covariate adjustment

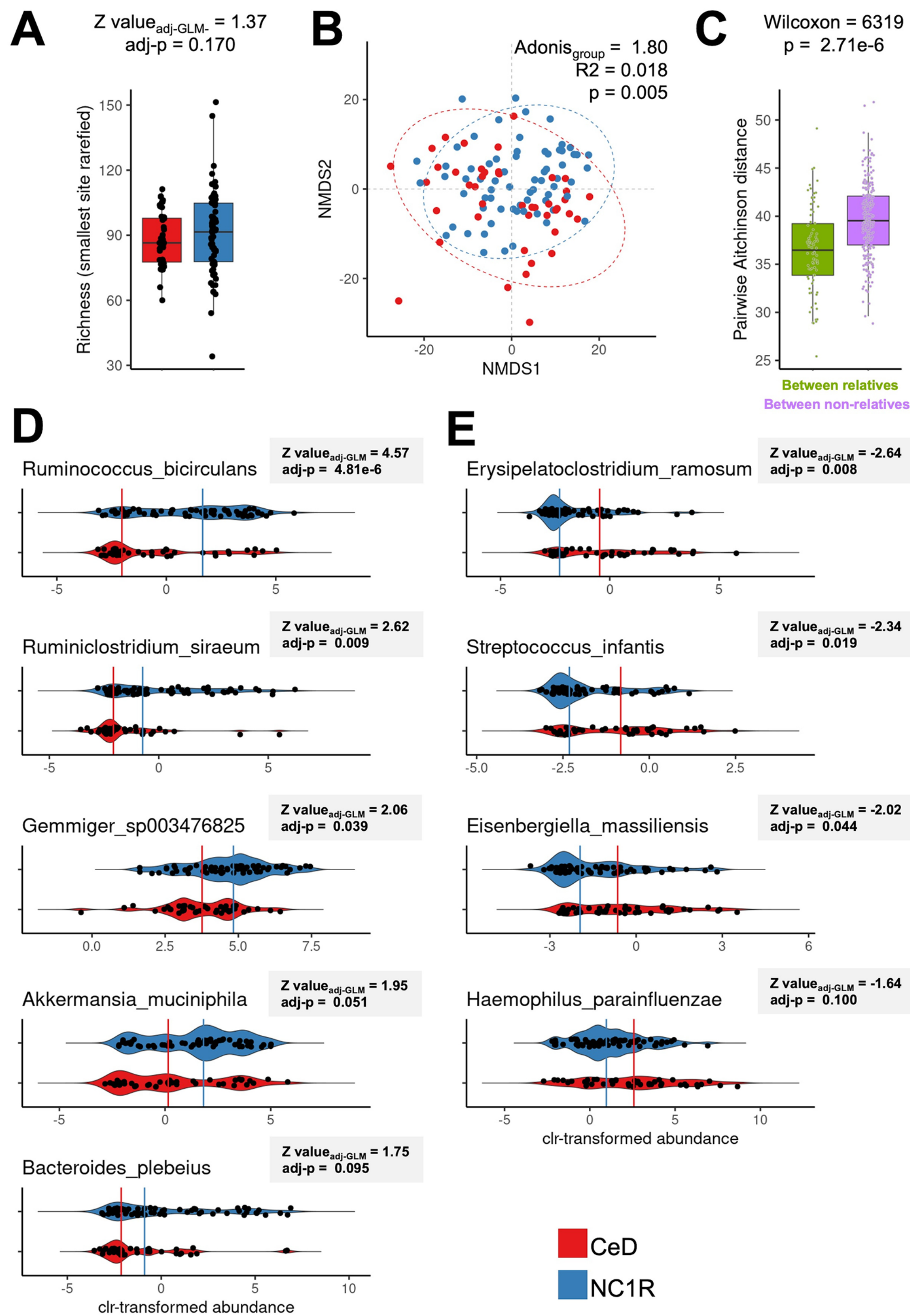

Fig. 2 (See legend on previous page.)

degradation, such as *Ruminococcus*, former *Eubacterium*, and *Prevotella* species. Among those taxonomy categories with reliable species delimitation and larger size-effect (GLM estimate), we found that *Ruminiclostridium siraeum* (adj-GLM = 0.87, Z-value = 2.62, adj-p = 0.009) and *Ruminococcus bicirculans* (adj-GLM = 17.5, Z-value = 4.57, adj-p = 4.81e-6) were more abundant in NC1R than in CeD. Besides, we also found that *Bacteroides plebeius* (adj-GLM = 0.47, Z-value = 1.75, adj-p = 0.095), *Akkermansia muciniphila* (adj-GLM = 0.67, Z-value = 1.95, adj-p = 0.051) and *Gemigger* sp. 003476825 (adj-GLM = 1.01, Z-value = 2.06,

adj-p = 0.039) were more abundant in control individuals (Fig. 2D). Conversely, CeD subjects exhibited a meaningful increased abundance of *Erysipelatoclostridium ramosum* (adj- adj-GLM = − 1.01, Z-value = − 2.64, adj-p = 0.008), *Haemophilus parainfluenzae* (adj-GLM = − 1.08, Z-value = − 1.64, adj-p = 0.100), *Eisenbergiella massiliensis* (adj-GLM = − 0.71, Z-value = − 2.02, adj-p = 0.044), and *Streptococcus infantis* (adj-GLM = − 0.81, Z-value = − 2.34, adj-p = 0.019) in their intestinal tract (Fig. 2E).

Microbiota and host variable data integration

CeD patients are prone to nutritional deficits because they are forced to adopt lifelong gluten-free diet regimes [22, 23]. Given the interrelated set of microbial species lessened in CeD fecal samples, we wondered if dietary patterns could directly influence these observations. Dietary records (categorical variables) for servings, whole grain, animal protein, dairy products, refined sugars, alcohol, coffee, vegetables, and fruit averaged daily intake were surveyed and integrated with microbiota data via logistic regression with covariate control to look for possible interactions explaining differential microbiota abundance. We detected several interactions among microbial species, exhibiting disparate abundance between study groups and certain diet variables obtained from self-reported records. Among them, *R. siraeum* seemed to be greatly influenced by several dietary parameters, which also differ between CeD and their NC1R. As a consequence, consumption of nutritional supplements (e.g. vitamins, minerals, and weight-loss supplements) negatively affected the abundance of *R. siraeum* (OR = 0.53, p = 0.002), whose higher intake was linked to CeD subjects (Chi-test = 11.3, p = 0.002) (Fig. 3A). Also, we revealed that *R. siraeum* was positively influenced by vegetable intake patterns. In particular, the intake of 3 servings a day boosted its abundance (OR = 1.27, adj-p = 0.015). Moreover, CeD subjects had a drastic drop of this microbial species in lower vegetable intake regimes (Fig. 3B). In this last regard, this association was reversed when vegetable servings a day was less than 3 (OR = 0.82, adj-p = 0.021), thus suggesting a direct nutrient-microbe interaction, with a larger effect on NC1R (*R. siraeum* median clr-abundance = − 1.25 vs 1.18 for low and higher vegetable intake, respectively, adj-p = 0.049).

On the other hand, we found several species differentiated between CeD subjects and NC1R to be strongly influenced by the number of servings consumed daily.

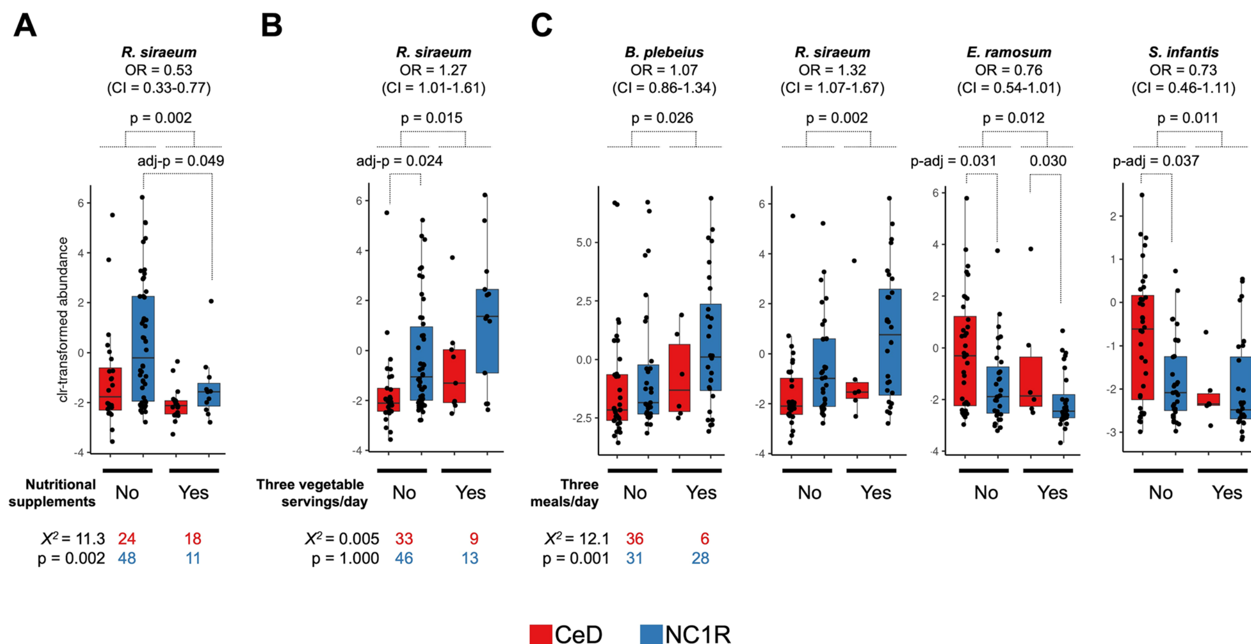

Fig. 3 Integration of gut microbiota data with host variables. Logistic regression (*stats::glm* function with binomial distribution assessment) with covariate control was used to evaluate correlation between dietary patterns and disparate gut microbiota traits between celiac (CeD) (red) and non-celiac 1st-degree relatives (NC1R) (blue). Statistical assessment supports interaction between *Ruminiclostridium siraeum* and nutritional supplementation (**A**), and with vegetable consumption (**B**). **C** The pattern of 3 servings a day was correlated with 4 out of 9 categories detected to be differentially abundant between CeD and NC1R (Fig. 1D, E). Comparison between subgroups was achieved using Wilcoxon Rank Sum test with multiple testing (FDR). Chi-squared test (*stats:chi.sq* function with Monte Carlo simulation) was computed to evaluate contingency tables resulting from subject grouping and dietary patterns as categorical and dichotomous variables

Again, *R. siraeum* (OR = 1.32, adj-p = 0.002) and, to a lesser extent, *B. plebeius* (OR = 1.07, adj-p = 0.025) were positively correlated with the consumption of 3 servings a day, a pattern more prevalent in NC1R (Chi-test = 12.1, p = 0.001). By contrast, this meal pattern seemed to have the opposite effect on *E. ramosum* (OR = 0.76, p = 0.012) and *S. infantis* (OR = 0.73, p = 0.011) abundance (Fig. 3C). Strikingly, this correlation pattern was fully reversed when considering intake regimes of more than 3 servings consumed daily, an observation that can plausibly link the abundance of such species with daily calorie intake. The regime of more than 3 servings a day was more frequent in CeD subjects (Chi-test = 11.3, p = 0.003), but CeD individuals had statistically significantly lower BMI compared to NC1R (20.9 vs 25.3, t = − 5.42, p = 4.49e-7), a surrogate marker for calorie intake. The above may be explained by the nutrient malabsorption characteristic of CeD [22–24]. However, it is important to notice that CeD subjects who consumed more than 3 servings a day (CeD, n = 35) had, on average, a BMI almost 1 unit higher than those who took lower number of servings per day (CeD, n = 7) (21.1 vs 20.4, no statistically significant difference); what might support the microbe-caloric intake interaction predicted. In NC1R the BMI average was equal in both dietary regimes (25.25 vs 25.31). Finally, we detected a negative association between fat consumption and *Akkermansia muciniphila* abundance. This species was lower in subjects consuming at least 3 servings/portions of fats/oils daily (OR = 0.74, CI 0.52–0.99, adj-p = 0.043), although no differential pattern of consumption was observed between groups (Chi-test = 0.06, p = 1.000). All in all, we cannot disregard the impact of nutrient quality, rather than quantity, on the microbial community structure and disease progression, given the radically different dietary regime regularly followed in CeD [25]. Nevertheless, this is still a limitation of the current study that make impossible to establish more specific diet-host-microbe interactions.

Discussion

Affecting approximately 1% of the world population [26], CeD remains a significant health concern, specifically because its incidence has been increasing over the past four decades [27]. In this study, we observed disease sex-association and diagnostic window of CeD patients in line with the literature [28]. Our findings underscore a sex disparity, with higher prevalence of CeD in women and heterogeneity in the age of CeD diagnosis indicating different clinical trajectories and possible diagnostic challenges in CeD [28, 29]. Both CeD patients and NC1R presented comorbidities that underlie the systemic nature of CeD and its association with the presence of autoimmune and non-autoimmune conditions, such as autoimmune thyroiditis and respiratory and/or allergic diseases [30].

In current study, mean BMI was higher in NC1R compared with CeD, concurrent with available literature [31]. Improvements on this biomarker were observed on a CeD population after 2.8 years on a gluten-free diet following diagnosis [32]. However, a lower BMI in CeD patients might also be attributed to a combination of malabsorption, chronic inflammation, and dietary restrictions [25]. We also observed that most individuals with CeD reported consuming more than 3 servings per day when compared with NC1R, as well as taking more nutritional supplements, which might be attributed to commonly observed nutritional deficiencies in CeD [22, 23]. Nevertheless, it is noteworthy that the majority of CeD subjects who reported intake of nutritional supplements in the current study were not followed by a nutritionist, which could emphasize the necessity for enhanced nutritional counseling to optimize CeD subjects' dietary management [25].

Given the tight interactions between the affected organ and microbes, gut microbiota assessments are frequent in CeD. Mostly, such studies try to find predictive biomarkers and seeking disease causal relationships in bacterial groups. However, given the technical limitations of the predominantly methodology (short-read based sequencing) to assess gut microbiota [33], no precise links between gut bacterial species and CeD have yet been stated [34]. As a result, taxonomy inventories predominantly highlight the differential abundance of Pseudomonadota (former Proteobacteria), Bacteroidota (former Bacteroidetes) and Bacilliota (former Firmicutes) phyla in a generic manner, disregarding the microbial complexity inside those bacterial taxa [3]. A recently published population-level analysis using whole-genome human genetics (SNPs) identified links between CeD and gut microbiota at the family level, suggesting potential causality on Ruminococcaceae and Lachnospiraceae taxa [35], a fact shedding no light on specific bacterial signatures either. In this last regard, CeD genetic risk (HLA-DQ genotype) has been used as a proxy to anticipate CeD onset in infants and concomitantly identify individual gut microbiota species contributing to disease development. In that way, pathogenic bacteria such as *Clostridioides perfringens* and *Clostridioides difficile* were more abundant in infants at genetic risk of CeD fed by formula milk [12]. Similarly, *Bifidobacterium longum*, a well-recognized beneficial species, has been proposed as a protective factor by their higher abundance in healthy controls compared to infants at CeD genetic risk [14]. Notwithstanding, the ample technical and methodological heterogeneity of studies impedes evidencing any consensus

gut microbiota signature in CeD and concrete species linked to that [36].

Advanced human microbiome research indicates high inter-individual variability but common signatures in relatives resulting from cohabiting and shared environmental exposure [37]. Therefore, our kinship study framework was pivotal in unveiling risk and protective gut microbiota traits in CeD by comparing subject groups sharing genetic and environmental features. Also, the cost-effective methodology used, based on third-generation sequencing technology with the potential to delineate microbial communities at the species level [38], was helpful in accurately defining such microbiota traits. Here, we described CeD subjects might be prone to loss of microbial species (richness) in their gut, an alpha diversity descriptor globally linked to health, and dietary diversity [39]. From the community structure level, such observed slight changes in diversity could drive differential community composition in CeD, detected by co-abundance and co-occurrence patterns explaining metabolic interactions. Furthermore, we found that microbial communities of relatives are significantly more similar when compared with non-relatives. Altogether, the gut microbiota patterns profiled here make it plausible to focus on disparate species between relatives as potential traits influencing disease progression.

We employed advanced methods for strict covariate control when trying to disclose such bacterial species. Outstandingly, our results partially replicated an independent study on another European population (Italy) using advanced shotgun metagenomics and pointing out the same abundance shifts for *Ruminococcus bicirculans* and *Haemophilus parainfluenzae* species when comparing CeD subjects with non-celiac counterparts and considering strict or relaxed adherence to a gluten-free diet [40]. Besides, another similar work with NC1R and using short-read based DNA sequencing technology also showed reduced abundance of *Akkermansia* species in CeD feces samples [41]. Consequently, our results are reinforced by previous clinical assessments using different experimental approaches, which make the associations between diet, microbiota, and the disease described here more likely.

From the microbiological point of view, the species attenuated in CeD seem to be central to digestion and production of microbiota-derived effector metabolites that guide intestinal function properly. For instance, *R. siraeum* is linked to resistant starch degradation [42] and is pointed out as a potential short-chain fatty acid (SCFA) producer, which can explain its role in controlling glucose metabolism [43] and reduce BCAA (branched-chain amino acids) levels in type 2 diabetes [44]. Moreover, this species appears to be attenuated in Crohn's disease as well [45]. On the other hand, *R. bicirculans* has been described as having the genome capacity to degrade plant glucans [46], like hemicelluloses, and to respond positively to tannins supplementation [47]. Its genome content makes it related to other fiber-degrading ruminococci [48]. As a result, the link between the above species and complex carbohydrates supports the diet-microbe interactions established for them here. In addition, regarding *R. bicirculans*, its abundance and function linked to the fermentation of plant-based complex carbohydrates seem beneficial for responders to cancer immunotherapy [49].

In a similar manner to ruminococci species described above, *B. plebeius* also appears as a fiber degrader by its genome-encoded capacity to degrade marine and terrestrial plant glycans [50–52]. This species, prevalent in Japanese natives in response to sushi intake (seaweeds) [50], improves the gut barrier in a chronic kidney disease animal model and reduces muscle atrophy [53]. Lastly, the lower abundance of *A. muciniphila* has been associated with multiple diseases in pre-clinical and clinical assessments. Classically, *A. muciniphila* depletion is linked to obesity and metabolic dysfunction of type 2 diabetes [54, 55]. However, recent reports suggest a pivotal role of *A. muciniphila* in hepatic steatosis, intestinal inflammation, and colon cancer [56]. Nowadays, a vast amount of scientific evidence points out that *A. muciniphila* is a direct contributor to maintaining the gut barrier and modulating immunity, thus reducing inflammation, which is the underlying cause of numerous diseases, including CeD.

Overall, we have detected the abundance loss of crucial species in CeD compared to their NC1R. Those species are associated with complex carbohydrate processing and modulate the immune system directly or via effector molecules, like SCFAs, produced as a consequence of fiber degradation [57]. Far from expecting to establish a causal link with the disease, we present evidence on the likely origin of such disparate microbial profiles that can contribute to disease worsening. Altered dietary patterns in CeD are well-known [25], but their impact beyond potential malnutrition has not been elucidated and can involve serious alterations of the gut physiology at the molecular level, which is not assessed clinically. Despite the limitation of our study to determine particular ingredients and quantify macronutrient intake in a detailed manner, the global microbiota evaluation in CeD and NC1R plausibly suggest CeD subjects may have a drastic reduction in their fiber intake, thus explaining the lower abundance of complex carbohydrate degraders [58]. The loss of fiber fermenters/degraders will cause less production of SCFAs that, in addition to being colonocyte fuel for their basic functioning [59], is critical to reducing inflammation

at different molecular levels [60–62]. Therefore, adopting a diet high in gluten-free fiber (e.g. sorghum, corn, oats, and rice derived ingredients) could help improve bowel movements, reinforce the gut barrier, and reduce inflammation [63], among other features that permit ameliorating CeD patients' quality of life.

Conclusions

The kinship perspective in this cross-sectional study of CeD subjects has evidenced the loss of fiber-foraging species in their gastrointestinal tract. Far from establishing a causal relationship, our results highlight that gluten-free dietary patterns required to be adopted by CeD as primary and single therapy for disease treatment can have important side effects affecting gut physiology. CeD subjects are susceptible to a decline in their fiber intake and fermenters symbionts, restricting the benefits of the effector molecules they produce as end-products of complex carbohydrate and anaerobic metabolism. Gluten-free fiber alternatives must be provided and recommended to CeD patients as a strategy to ameliorate their quality of life, until a cure for the disease is found.

Supplementary Information

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Additional file 1

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Author contributions

Conceptualization, SGP; data curation, AR, JZ and AB-P; formal analysis, AR, AB-P and SGP; funding acquisition, SGP; investigation, AR, AB-P and SGP; methodology, AR, JZ, SBr, MBT, AB-P and SGP; project administration, SGP; resources, SBr, MBT, AG, SBa, AB-P and SGP; software, AB-P; supervision, SGP; validation, AB-P and SGP; visualization, AR, AB-P and SGP; writing—original draft, AR and AB-P; writing—review and editing, AB-P and SGP. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The raw fast5 data generated from rrn sequencing can be accessed at the European Nucleotide Archive via the bioproject accession number PRJEB74092.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Leiria Hospital Center. Informed consent was obtained from the participants or legally authorized representative for their anonymized information to be published in this article.

Consent for publication

Informed consent for publication was obtained from the participants or legally authorized representative.

Competing interests

The authors declare no competing interests.

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