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Salivary Gluten Degradation and Oral Microbial Profiles in Healthy Individuals and Celiac Disease Patients

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ABSTRACT Celiac disease (CD) is a chronic immune-mediated enteropathy induced by dietary gluten in genetically predisposed individuals. Saliva harbors the second highest bacterial load of the gastrointestinal (GI) tract after the colon. We hypothesized that enzymes produced by oral bacteria may be involved in gluten processing in the intestine and susceptibility to celiac disease. The aim of this study was to investigate salivary enzymatic activities and oral microbial profiles in healthy subjects versus patients with classical and refractory CD. Stimulated whole saliva was collected from patients with CD in remission ($n = 21$) and refractory CD (RCD; $n = 8$) and was compared to healthy controls (HC; $n = 20$) and subjects with functional GI complaints ($n = 12$). Salivary gluten-degrading activities were monitored with the tripeptide substrate Z-Tyr-Pro-Gln-pNA and the α -gliadin-derived immunogenic 33-mer peptide. The oral microbiome was profiled by 16S rRNA-based MiSeq analysis. Salivary glutenase activities were higher in CD patients compared to controls, both before and after normalization for protein concentration or bacterial load. The oral microbiomes of CD and RCD patients showed significant differences from that of healthy subjects, e.g., higher salivary levels of lactobacilli ($P < 0.05$), which may partly explain the observed higher gluten-degrading activities. While the pathophysiological link between the oral and gut microbiomes in CD needs further exploration, the presented data suggest that oral microbe-derived enzyme activities are elevated in subjects with CD, which may impact gluten processing and the presentation of immunogenic gluten epitopes to the immune system in the small intestine.

IMPORTANCE Ingested gluten proteins are the triggers of intestinal inflammation in celiac disease (CD). Certain immunogenic gluten domains are resistant to intestinal proteases but can be hydrolyzed by oral microbial enzymes. Very little is known about the endogenous proteolytic processing of gluten proteins in the oral cavity. Given that this occurs prior to gluten reaching the small intestine, such enzymes are likely to contribute to the composition of the gluten digest that ultimately reaches the small intestine and causes CD. We demonstrated that endogenous salivary protease activities are incomplete, likely liberating peptides from larger gluten proteins. The potentially responsible microbes were identified. The study included refractory CD patients, who have been studied less with regard to CD pathogenesis.

KEYWORDS 16S rRNA, celiac disease, enzyme, microbiome, saliva

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Celiac disease (CD) is a T-cell-mediated inflammatory enteropathy characterized by villous atrophy and crypt hyperplasia resulting from duodenal inflammation. The environmental trigger of CD is ingested gluten, a heterogeneous mixture of glutamine- and proline-rich proteins from wheat, rye, and barley. Untreated CD is characterized by autoantibodies to tissue transglutaminase (TG2), an (intestinal) enzyme that binds and modifies gluten peptides, resulting in their improved binding to HLA-DQ2 or HLA-DQ8, the major genetic predisposition for CD, followed by activation and expansion of destructive Th1 T cells in the gut (1). Apart from the established roles of gluten, HLA-DQ2/HLA-DQ8, and TG2, environmental factors such as viral and microbial infections and perhaps feeding practices have also been suggested to contribute to CD pathogenesis (2–6).

With regard to environmental factors, much attention has been directed at the endogenous microbiome and/or infections that may precipitate in the loss of tolerance to gluten. Gut microbial compositions differ in patients with CD compared to healthy subjects (7). For example, increased levels of potentially harmful *Bacteroides* and virulent *Escherichia coli* and decreased levels of commensal bifidobacteria have consistently been found in the duodenum and feces of CD patients, regardless of disease activity (7–11). In another study, increased proportions of enterobacteria and staphylococci were reported in CD patient fecal samples and duodenal biopsy specimens, and a gluten-free diet (GFD) restored these proportions to the levels of healthy individuals (8). However, it remains to be shown if these microbial changes drive disease activity or are merely a consequence of the ingested gluten-containing food and intestinal inflammatory activity. Additionally, very little is known about the mechanistic aspects of the transition from CD to refractory CD (RCD), a condition where disease persists despite the strict avoidance of gluten, and changes in the oral-gastrointestinal (GI) microbiome that may contribute to the persistent inflammatory status in RCD.

Most microbiome research in CD has been limited to the role of the intestinal microbiota. The oral cavity is part of, and in direct connection with, the entire gastrointestinal tract. Salivary microbial analysis thus can be considered a complement to duodenal and fecal microbial analysis to capture disease-specific microbial changes along the entire gastrointestinal tract. Approximately 1 liter of saliva, containing a diverse collection of aerobic and anaerobic bacteria, is swallowed on a daily basis, emphasizing the physical connection between the upper and the lower gastrointestinal tract (12). The estimated numbers of bacteria in dental plaque and saliva are 10^{11} per gram of dental plaque and 10^8 per ml of saliva (13–15), making the oral cavity the second most densely colonized part of the human digestive tract after the colon. In addition, saliva contains a wide variety of species (16) that differ distinctly from the communities in the gut (17).

We recently reported that select oral bacterial species can cleave gliadins, the ethanol-soluble fraction of gluten, in regions that remain incompletely digested by mammalian digestive enzymes (18–21). For example, the protease-resistant highly immunogenic 33-mer α -gliadin peptide (22) can be completely degraded by dental plaque bacteria. This led us to speculate that these salivary activities differ in healthy patients and those with CD. Gluten metabolisms in patients with CD, and the role of the endogenous microbiome in this process, have received renewed attention since the discovery of elevated fecal protease activities in CD (23). More recently, an *in vivo* study in mice populated with human intestinal bacteria showed that the microbiome of CD patients processes gluten differently compared to the microbiome of healthy subjects (24). Oral gluten digestion occurs prior to the gluten metabolites reaching the CD-affected duodenum, and therefore, in the present study, we quantified the endogenous gluten-degrading protease activities in saliva from healthy subjects and CD and RCD patients. Furthermore, the oral microbiomes were compared, aiming to identify disease-specific signatures and/or pathogenic links.

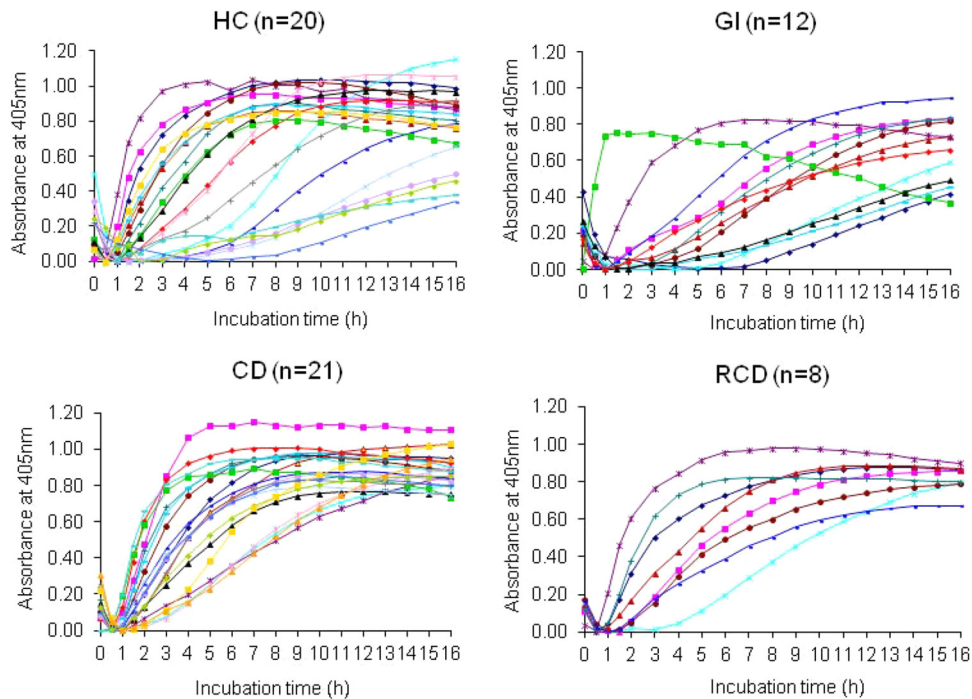


FIG 1 Rates of Z-YPQ-pNA substrate hydrolysis by enzymes in whole saliva (WS) from HC, GI, CD, and RCD patients. Aliquots of 200 μ l WS from each patient were incubated with 200 μ M Z-YPQ-pNA at 37°C. Substrate hydrolysis was monitored spectrophotometrically at 405 nm. Plotted are the averages of three kinetic curves obtained for each individual. Rates varied on average 10.6% within subjects.

RESULTS

Demographic information and whole saliva sample characteristics. The clinical information of the donors is shown in Table S1 in the supplemental material, and the demographic information of the donors and the whole saliva (WS) characteristics are summarized in Table S2 in the supplemental material. Healthy controls (HCs) were matched to CD patients in gender and age. RCD is commonly diagnosed in older individuals (25), which is reflected by the higher average age in this group. No differences were found between groups for the stimulated WS flow rate. The average protein levels were determined in the supernatant and were higher in the RCD group than in the HC group ($P = 0.022$). The relative total bacterial load, as determined by quantitative PCR (qPCR) with universal 16S rRNA genes primers, was statistically similar between groups, although the CD group average trended to be higher.

Hydrolysis of gluten substrates in human saliva. Z-YPQ-pNA was hydrolyzed in all of the subjects' saliva, albeit at different rates (Fig. 1). No hydrolysis was observed in boiled WS (data not shown). All kinetic plots trended toward a plateau optical density at 405 nm (OD_{405}) of 0.9 at which all substrate is hydrolyzed. From the kinetic plots, the half-life ($t_{1/2}$) values were determined, which are inversely related to the rates of substrate hydrolysis (Fig. 2). To reduce the number of statistical comparisons (see the Materials and Methods section), the initial analyses contrasted CD patients (CD and RCD) with non-CD participants (HCs and subjects with GI complaints; *post hoc* analyses confirmed that these two groups did not differ, $P > 0.10$). As shown in Fig. 2A, the $t_{1/2}$ value in the CD patient group was significantly lower, and thus the degradation rate was higher, than that in the non-CD controls. These differences remained after normalization for total protein (Fig. 2B) or total bacterial load (Fig. 2C).

Degradation of the immunogenic gliadin-derived 33-mer in WS was studied by reversed-phase high-performance liquid chromatography (RP-HPLC). This was feasible since the 33-mer elutes at around 66 min, whereas the majority of the WS salivary proteins elute between 27 and 64 min at the applied gradient (see Fig. S1 in the supplemental material). The 33-mer peak heights as a function of incubation time for

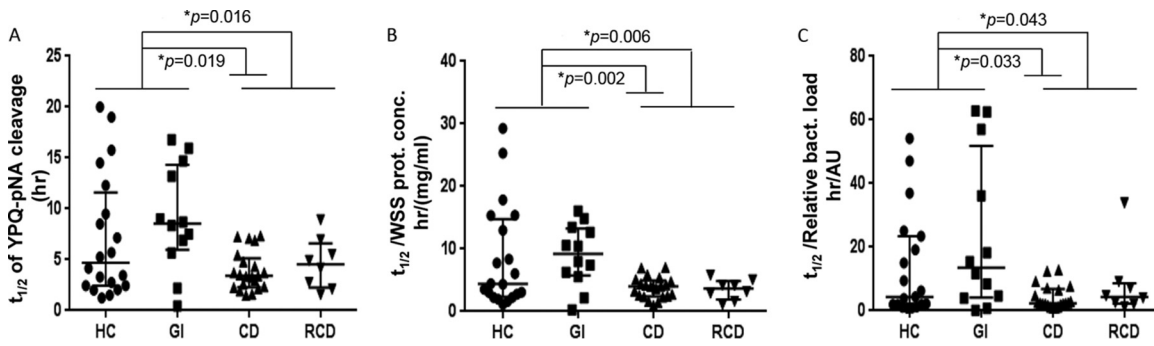


FIG 2 Half-life ($t_{1/2}$) values of Z-YPQ-pNA hydrolysis in whole saliva (A), normalized for total salivary protein concentration (B) and total salivary bacterial load (C). The values were determined from plots shown in Fig. 1. Vertical bars indicate medians and interquartile ranges.

all subjects are shown in Fig. 3. The $t_{1/2}$ values, calculated from these plots, showed that they were significantly lower in the CD group than the controls (i.e., HCs and subjects with GI complaints combined) (Fig. 4A), and this difference remained after normalization for total protein or total bacterial DNA (all $P < 0.05$) (see Fig. 4B and C, respectively). When the patients (CD or RCD) were compared with the controls (HCs and subjects with GI complaints), the pattern of results was essentially comparable to the observations made with the Z-YPQ-pNA substrate, although P values now bordered on statistical significance ($P = 0.055$). Overall, the $t_{1/2}$ values for the Z-YPQ-pNA and 33-mer substrates showed a consistent pattern of enzyme activity in WS being modestly but significantly elevated in the CD groups, before and after normalization for total protein concentration or bacterial load.

Salivary microbiome in HC, GI, CD, and RCD subjects. The microbial composition in the WS samples was analyzed by 16S rRNA gene analysis. On average, 64,223 reads were generated for each subject’s saliva sample (see Table S3 in the supplemental material). The operational taxonomic unit (OTU) tables are shown in Table S4 in the supplemental material. The salivary microbiota were first compared at the phylum level (Fig. 5A). Most of the oral bacteria detected belonged to the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria*. Among these, *Bacteroidetes* and *Firmicutes* were found to comprise ~60% of the total oral microbes, consistent with

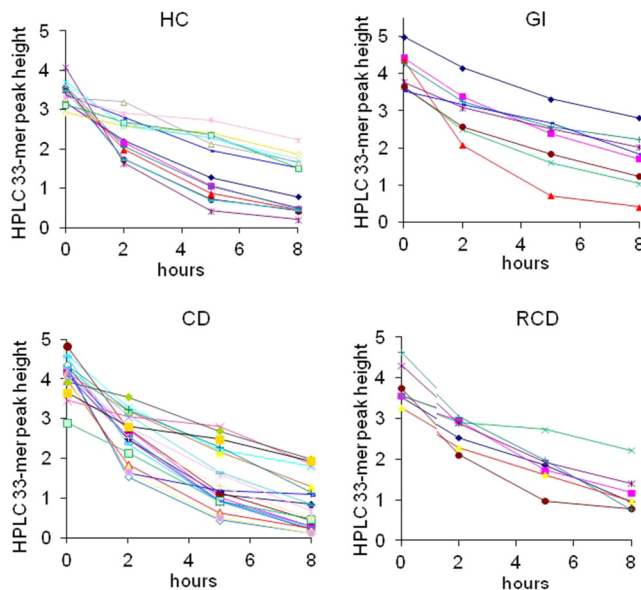


FIG 3 RP-HPLC analyses of the degradation of the 33-mer gliadin peptide in WS from all subjects. Plotted are the 33-mer peak heights versus the incubation time (0, 2, 5, and 8 h) in WS.

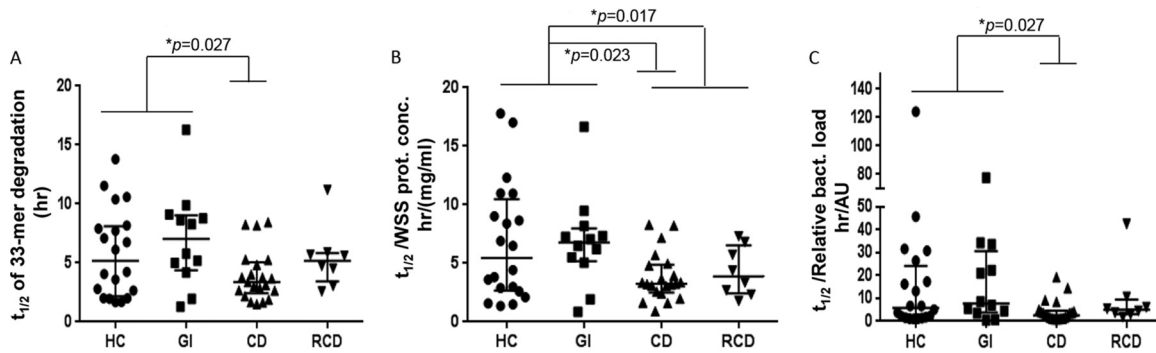


FIG 4 Half-life ($t_{1/2}$) values of 33-mer hydrolysis in whole saliva (A), normalized for total salivary protein concentration (B) and total salivary bacterial load (C). The values were determined from plots shown in Fig. 2. Vertical bars indicate median values and interquartile ranges.

previous reports on the composition of the oral microbiome (26). Among the five major phyla, a few differences between groups were observed (see Fig. S2 in the supplemental material). The significant differences were between the CD and RCD groups with respect to *Bacteroidetes* (CD > RCD), *Actinobacteria* (CD < RCD), and *Fusobacteria* (CD > RCD). The overall microbial diversity was greater in the HC group than in the CD group ($P = 0.017$) (Fig. 5B). No differences were observed between non-CD control groups (HCs versus GI subjects; $P > 0.10$).

Analyses at the phyla level may obscure differences at the species level, and therefore these were analyzed separately. Differences at the species level were assessed by pairwise comparisons between selected groups (HC versus CD, HC versus RCD, GI subjects versus CD, GI subjects versus RCD, and CD versus RCD). These analyses

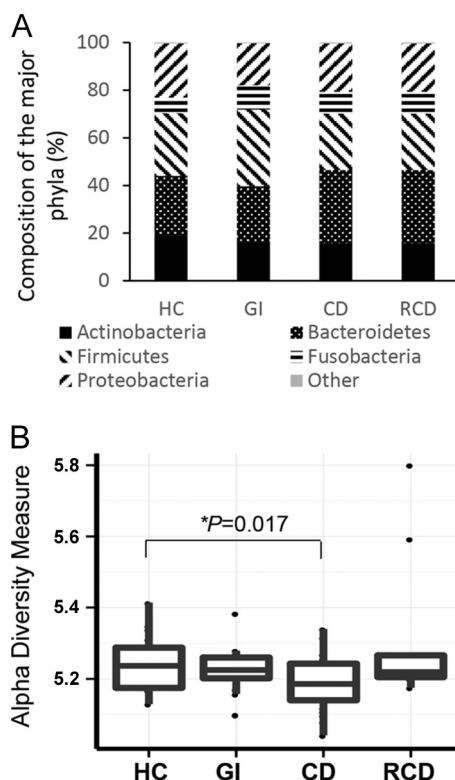


FIG 5 Composition and abundance of salivary bacteria in WS of HC, GI, CD, and RCD subjects. (A) Composition and relative abundance (percentage) of bacteria in WS detected at the phylum level. (B) Microbial diversity analysis between groups. The Shannon diversity index was calculated using the 16S microbial abundance data for each sample. The mean diversity was significantly higher in the HC compared to the CD subjects ($P = 0.016$, Welch's t test).

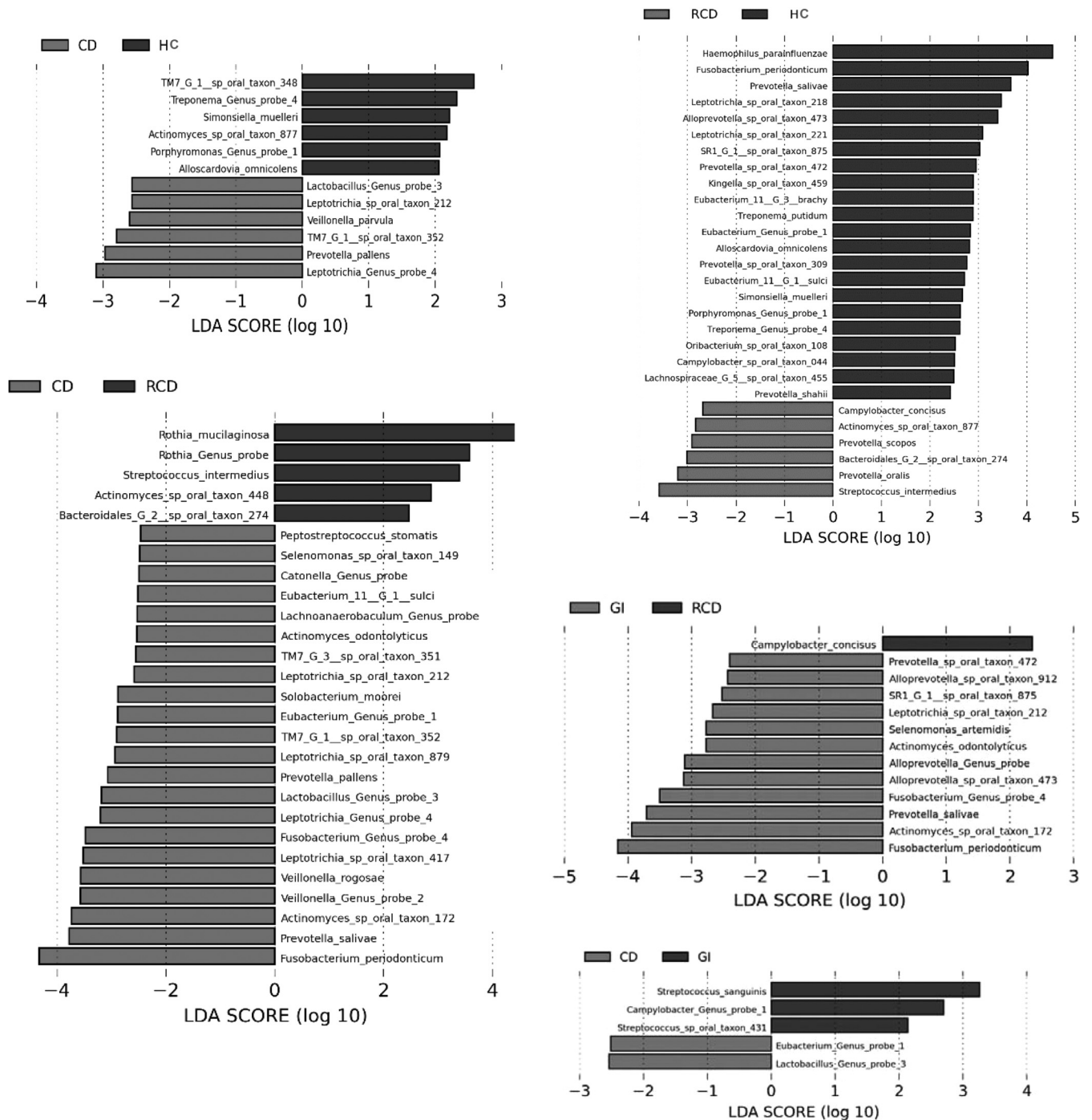


FIG 6 Oral species with significant differences between groups. Oral microbial communities in HC, CD, RCD, and GI groups were compared in a pairwise fashion, and potential microbial biomarker species indicative of one or the other condition were identified using the LefSe tool (see Materials and Methods). The longer a bar, the stronger the evidence that the corresponding organism may act as a biomarker in the given condition. Absolute LDA scores greater than 2.0 were considered significant.

revealed differences between groups. Species that exhibited significantly ($P < 0.05$) increased levels in each patient group are shown in Fig. 6, and more detailed species information is provided in Table S5 in the supplemental material. In the HC versus CD as well as the HC versus RCD comparisons, the HC group was found to harbor consistently higher levels of *TM7 sp.*, *Treponema sp.*, *Simonsiella muelleri*, *Actinomyces sp.*, *Porphyromonas sp.*, and *Alloscardovia omnicolens*. CD and RCD are distinctly different disease entities according to their oral microbial signatures as shown in a pairwise

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comparison. In CD, 22 species were elevated compared to RCD, with almost half of them belonging to the *Firmicutes* phylum. In RCD, five species showed higher proportions compared to CD, most belonging to the *Actinobacteria* phylum.

DISCUSSION

In this study, we investigated salivary protease activities and microbial profiles given their potential involvement in gluten digestion and processing in CD. The first finding was that gluten degradation rates were significantly higher ($t_{1/2}$ values lower) in saliva from CD patients, regardless of whether the tripeptide substrate or the 33-mer substrates were employed. The lower $t_{1/2}$ values remained statistically significant after correction for total protein concentration and total bacterial load. Early on, it was hypothesized that CD is a digestive disorder that is caused by enzyme deficiencies (reviewed in reference 27). Our new findings in saliva, and the previous work on feces (23, 24), show enhanced and altered bacterial gluten degradation in CD, suggesting that enzymatic processing may play an auxiliary role in CD pathogenesis. Gluten processing by microorganisms colonizing the oral cavity may be particularly relevant. While the residence time of ingested gluten in the oral cavity is short, saliva contains a significantly higher number of microbes and a wider variety of species than those colonizing the stomach and the duodenum. Moreover, the oral microbial processing of gluten, perhaps continued to some extent in the stomach due to swallowed saliva, takes place upstream from the duodenum where CD manifests.

The observed rates of gluten substrate degradation in saliva were relatively low, and it can be readily concluded that the endogenous oral microbiome would be incapable of fully digesting gluten-containing meals. Partial digestion may be highly relevant, however, from the perspective of how gluten is being presented further downstream in the gastrointestinal tract. Recently, it was demonstrated in a CD mouse model that gluten was partly digested by *Pseudomonas aeruginosa*, which liberated smaller peptides that were then more easily translocated through the mouse intestinal barrier and fueled disease activity (24). Analogously, oral microbial enzyme activities may likewise contribute to liberating more immunogenic gluten peptides. In support of this, *in vitro* studies with suboptimal doses of the gluten-degrading enzymes EP-B2 and AN-PEP have demonstrated that incomplete digestion leads to the potentiation of gluten immunogenicity, rather than a reduction, through the generation of more peptides with immunostimulatory potential (28, 29). Therefore, an excess of minor, incomplete digestions carried out by certain endogenous oral and duodenal microbiomes may increase the load and transepithelial transport of active immunogenic gluten epitopes in the duodenum and, therefore, promote CD activity, a mechanism which should be further explored.

With regard to the salivary gluten-degrading activities among groups, they remained significant after normalization for total bacteria, suggesting that the saliva of CD patients was enriched with oral bacteria capable of gluten degradation. The bacterial species that were significantly higher in CD and that may have contributed to the increased gluten degradation in CD are *Lactobacillus* species (30). Lactobacilli are natural colonizers of the oral cavity, with *Lactobacillus rhamnosus* being the most prevalent (26). While several studies have shown that the levels of *Lactobacillus* were lower in the duodenal and fecal samples in CD patients compared to healthy controls (31–33), reports on *Lactobacillus* levels in the oral cavity are inconsistent (34–36). Regardless of the microbial source, the modest increase in salivary enzyme activity is likely to be derived from bacteria given that dental plaque is a rich source of gluten-degrading activity (21).

At the phylum and species levels, the oral microbiome of patients with RCD was clearly and significantly different from that of patients with CD. The differences between the CD and RCD groups provided evidence that, from an oral microbial perspective, CD is unlike RCD, suggesting that, as CD progresses to RCD, oral microbial colonization patterns change. These changes may offer new opportunities for screening and diagnosis and potentially evaluate the risk of a CD patient transitioning to RCD.

However, retrospective and prospective follow-up studies are needed to confirm this assumption.

There are some limitations to our study. For instance, all CD patients enrolled were on a gluten-free diet. While the influence of a gluten-free diet on oral microbial colonization has not been well characterized, there is a report where the change from one type of gluten-free diet to another affected the oral microbiome (37). Oral hygiene effects in our study were minimized by exclusion of subjects with overtly poor oral hygiene following dental examination. Other factors that may have affected the outcomes of this study are higher prevalence of IgA deficiency in the CD and RCD groups (38). While the latter may be excluded in our study, both primary disease-related and secondary disease-associated factors together likely contribute to an environment in which oral bacterial gluten processing may be altered.

Overall, the contribution of oral microbial enzymes in the digestion of gluten and other dietary proteins has been a relatively unexplored area of investigation. With gluten as a paradigmatic dietary protein that can cause a specific gastrointestinal disorder, i.e., CD, further studies on understanding the digestive proteolytic processing of gluten and other proteins, including nutritional allergens (39), are feasible and warranted. These should take into account the totality of enzymatic activities acting on gluten as it passes from the oral cavity to the duodenum, mediated by microbial as well as mammalian digestive enzymes, where differences in the immunogenicity of the peptide pool generated can be assessed.

MATERIALS AND METHODS

Human subjects and inclusion/exclusion criteria. Prior approval for this study was obtained from the Committee for Clinical Investigations at the Beth Israel Deaconess Medical Center and the Institutional Review Board at Boston University. Stimulated whole saliva (WS) was collected from four groups of subjects, comprising two patient groups and two control groups (see Table S1 in the supplemental material) (40, 41). The two patient groups comprised (i) CD patients who had presented with positive anti-deamidated gliadin peptide (anti-DGP) and/or anti-TG2 IgA antibodies (42) and duodenal villous atrophy at diagnosis and who had responded to a gluten-free diet (GFD) and were thus in remission (CD; $n = 21$) and (ii) RCD patients who were previously diagnosed with CD and had persistent or recurrent malabsorptive symptoms and persistent or recurrent villous atrophy on small bowel biopsy specimen despite a strict GFD for at least 6 months (43) (RCD; $n = 8$, protease studies; $n = 9$, microbiome studies; RCD type I except for 1 patient with RCD type II). The CD patients were on a gluten-free diet (GFD) for an average of 43 months and the RCD patients for 85 months at the time of the study. The two non-CD control groups comprised (i) healthy subjects (HC), displaying no signs (genetic, serological, or histological) or symptoms of CD or gluten sensitivity ($n = 20$) and (ii) patients reporting functional gastrointestinal (GI) complaints, i.e., nonimmune-mediated gastrointestinal symptoms without a known somatic cause and in which CD had been excluded by serological and histological testing ($n = 12$).

All subjects were at least 18 years of age and provided informed consent prior to participation in the study. The exclusion criteria for all groups were use of illicit drugs or excessive alcohol, having unstable heart disease, kidney disease, liver disease, or a clinically significant mental illness. Not excluded were patients suffering from diabetes (3 CD and 2 RCD patients). There was no antibiotic use, except for one CD patient taking rifaximin.

Whole saliva (WS) collection. All whole saliva samples were collected by a trained dentist, and oral health status was evaluated with a questionnaire. Those indicating dry mouth complaints or displaying overt signs of dental or periodontal health issues were excluded from the study. Donors refrained from eating, smoking, drinking (except water), use of a mouth rinse, and tooth brushing for at least 1 h prior to sample collection. They were then asked to rinse their mouth with water three times and were given a 20-cm by 20-cm Parafilm (Sigma, St. Louis, MO) for masticatory stimulation of salivary flow (44).

WS was collected into graduated 50-ml centrifuge tubes placed on ice, and the time for the collection of a 10-ml volume was recorded. After vortexing, the collected WS was separated into 1-ml aliquots, and half of the aliquots were centrifuged at $16,000 \times g$ for 10 min at 4°C to separate the WS cell-free supernatant from the WS pellet. All samples were immediately frozen at -80°C and stored until analysis.

Hydrolysis of Z-YQP-pNA in WS. Benzoyloxycarbonyl-Tyr-Pro-Gln-paranitroanilide (Z-YQP-pNA) was chemically synthesized at 95% purity (21st Century Biochemicals, Marlboro, MA), dissolved in 75% dimethyl sulfoxide to 10 mM, and added to the WS samples to a final concentration of 200 μM . Substrate hydrolysis was monitored at 405 nm every hour for 16 h. Each subject sample was tested in triplicate. The average maximum rate of hydrolysis was determined for each subject as well as the half-life ($t_{1/2}$) value, defined as the time needed to degrade 50% of the added substrate.

Degradation of the 33-mer in WS. The gliadin-derived immunogenic 33-mer peptide with the amino acid sequence LQLQPFPPQLPYQPQLPYQPQLPYQPQPF (22) was synthesized at a purity of $>90\%$ (21st Century Biochemicals, Marlboro, MA), which was confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC) (see below). The 33-mer peptide was added to WS to a final concentration of 250 $\mu\text{g/ml}$. After incubation for 0, 2, 5, and 8 h at 37°C , 100- μl aliquots were

removed and boiled for 10 min to deactivate enzymatic activity. Degradation of the 33-mer was monitored by using C_{18} RP-HPLC where fragments were eluted with a shallow gradient from 0% to 55% buffer B containing 80% acetonitrile and 0.1% trifluoroacetic acid over a 75-min time interval as previously described (19). Control experiments were conducted with boiled (inactivated) WS samples with added 33-mer ($n = 3$).

Microbial DNA isolation from WS. A 1-ml aliquot of WS was centrifuged, and bacterial DNA was isolated from the pellet with the MasterPure Gram-positive DNA purification kit (Epicentre, Madison, WI, USA) (45). An aliquot of 150 μ l of Tris-EDTA (TE) buffer, pH 7.5, containing Ready-Lyse Lysozyme was added, and samples were incubated for 16 h at 37°C. Subsequently, 150 μ l of proteinase K/Gram-positive lysis solution was added, followed by incubation at 65 to 70°C for 15 min. After cooling to room temperature, 175 μ l of protein precipitation reagent was added to 300 μ l of the lysed sample. The supernatant was obtained by centrifugation, and an aliquot of 1 μ l of RNase A (5 μ g/ μ l) was added followed by incubation at 37°C for 30 min. Then, 500 μ l of isopropanol was added, and the DNA was pelleted by centrifugation at 4°C for 10 min at $16,000 \times g$. The supernatant was removed, and the pellet was washed once with 70% ethanol and suspended in 50 μ l of 10 mM Tris-HCl (pH 8.0). The DNA concentration was determined using a NanoDrop 2000c (Thermo Scientific, Tewksbury, MA), and samples were kept at -20°C until analysis.

Quantitative (real-time) PCR. To compare the total salivary bacterial load among subjects, a universal primer set for the bacterial 16S rRNA gene was used with the following sequences: forward 5'-GTGSTGCAYGGYGTGTCGTC-3', 1048 to 1067; reverse 5'-ACGTCRTCCMCACCTTCCTC-3', 1175 to 1194 (46). The qPCR was carried out in triplicate in reaction volumes of 20 μ l with 10 μ l of 2 \times SYBR green select master mix (Applied Biosystems, Foster City, CA, USA), forward and reverse primers, each at a final concentration of 150 nM, and a 2-ng DNA template. The qPCRs were performed using the Applied Biosystems StepOnePlus real-time PCR system, applying the following conditions: uracil-DNA glycosylase (UDG) activation (50°C, 2 min); DNA polymerase activation (95°C, 2 min); denaturation (95°C, 15 s); and annealing/extending (60°C, 1 min). In each experiment, a nontemplate control was included. The expected size of the amplicon was 147 bp.

16S rRNA gene amplicon sequencing and analysis. Microbial diversity analysis in the WS pellet samples was studied by sequencing the amplified V3 to V4 hypervariable region of the 16S rRNA gene as described previously (45). In brief, WS pellet DNA (50 ng) was amplified with 341F/806R universal primers and 5 Prime HotMaster mix (5 Prime). The primer sequences were AATGATACGGCGACCACCGA GATCTACTACTATGGTAATTGCTCTACGGGAGGCAGCAG (341F; forward primer) and CAAGCAGAAGACGGC ATACGAGATNNNNNNNNNNAGTCAGTCAGCCGGACTACHVGGGTWCTAAT (806R; reverse primer, where the 12 N's designate barcode sequence). A total of 96 barcodes were used in the study. The PCR samples were purified using AMPure beads (Beckman Coulter, Milan, Italy) and then quantitated using the NanoDrop 2000c. Equal amounts of libraries were pooled (100 ng/library) and subjected to 2% agarose gel electrophoresis. Bands of \sim 590 bp were excised, and DNA was extracted with the Qiagen MinElute gel extraction kit. The library was analyzed on a Bioanalyzer Instrument (Agilent, Santa Clara, CA, USA) for quantitation. The quantified library was diluted to 12 picomolar following the Illumina protocol, spiked with 20% PhiX, and sequenced with MiSeq (Illumina) according to the manufacturer's recommendations. Raw sequences were generated by paired-end sequencing (2×250). Initial read quality was assessed using the FastQC quality control tool for high-throughput sequence data (available at <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The lengths of the generated reads were 250 bases for both forward and reverse sequences, and the overlapping portions of the paired reads were merged using FLASH sequence software (46), creating a high-quality longer read covering the entire V3 to V4 target region, generally about 440 bp. Community diversity profiles were developed using the QIIME analysis pipeline (47). Reads were clustered into OTUs through the open reference clustering pipeline. The reference HOMD database (48) along with a 97% identity threshold was used to guide the clustering of reads. To check for confounding chimeras in the OTUs, we used UCHIME (25) in combination with the "Gold" database (http://drive5.com/uchime/uchime_download.html).

Statistical analysis. SPSS 22.0 software was used for statistical analysis. Differences in Z-YPQ-pNA, 33-mer degradation rates, as well as microbial groups at the phylum level between groups were determined using Mann-Whitney U tests. To limit the number of statistical comparisons and type I error rate, analyses first contrasted non-CD controls (HC, GI subjects) with CD patients (CD, RCD), which was justified on *a priori* grounds and buttressed by the lack of statistical differences between HCs and GI subjects or CD and RCD (see Results). Only in case of significant differences between the two groups, further analyses continued to contrast HCs versus GI subjects and CD versus RCD, respectively. The level of significance was set at a P value of <0.05 .

For between group comparisons at the species level, the linear discriminant analysis effect size (LEfSe) method was used (47), which performs a linear discriminant analysis coupled with effect size measurements to identify organisms that may act as biomarkers in the microbial communities associated with the different groups. LEfSe was performed with default α values of <0.05 for the Kruskal-Wallis test and a linear discriminant analysis (LDA) score of 2.0 to determine discriminative features. To estimate taxonomic diversity for the different conditions, the Shannon diversity index was calculated for each sample using the 16S rRNA sequence data. Statistical significance was estimated using Welch's t test. All calculations were performed using the R programming language (R version 3.2.3) and the phyloseq package (phyloseq version 1.14.0) (48).

Accession number(s). Raw sequences analyzed in this project are available through the NCBI Sequence Read Archive under BioProject no. [PRJNA321349](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA321349).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.03330-16>.

TEXT S1, PDF file, 0.2 MB.

DATA SET S1, XLS file, 0.4 MB.

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We declare no conflicts of interest.

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