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Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity

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BACKGROUND & AIMS: Partially degraded gluten peptides from cereals trigger celiac disease (CD), an autoimmune enteropathy occurring in genetically susceptible persons. Susceptibility genes are necessary but not sufficient to induce CD, and additional environmental factors related to unfavorable alterations in the microbiota have been proposed. We investigated gluten metabolism by opportunistic pathogens and commensal duodenal bacteria and characterized the capacity of the produced peptides to activate gluten-specific T cells from CD patients. **METHODS:** We colonized germ-free C57BL/6 mice with bacteria isolated from the small intestine of CD patients or healthy controls, selected for their in vitro gluten-degrading capacity. After gluten gavage, gliadin amount and proteolytic activities were measured in intestinal contents. Peptides produced by bacteria used in mouse colonizations from the immunogenic 33-mer gluten peptide were characterized by liquid chromatography tandem mass spectrometry and their immunogenic potential was evaluated using peripheral blood mononuclear cells from celiac patients after receiving a 3-day gluten challenge. **RESULTS:** Bacterial colonizations produced distinct gluten-degradation patterns in the mouse small intestine. *Pseudomonas aeruginosa*, an opportunistic pathogen from CD patients, exhibited elastase activity and produced peptides that better translocated the mouse intestinal barrier. *P aeruginosa*–modified gluten peptides activated gluten-specific T cells from CD patients. In contrast, *Lactobacillus* spp. from the duodenum of non-CD controls degraded gluten peptides produced by human and *P aeruginosa* proteases, reducing their immunogenicity. **CONCLUSIONS:** Small intestinal bacteria exhibit distinct gluten metabolic patterns in vivo, increasing or reducing gluten peptide immunogenicity. This microbe–gluten–host interaction may modulate autoimmune risk in genetically susceptible persons and may underlie the reported association of dysbiosis and CD.

Keywords: Celiac Disease; Gluten Metabolism; Intestinal Microbiota; Intestinal Inflammation.

Gluten-related disorders are increasingly prevalent conditions[1] that encompass all diseases triggered by dietary gluten, including celiac disease (CD), a T-cell–mediated enteropathy, dermatitis herpetiformis, gluten ataxia, and other forms of non-autoimmune reactions.[2] Gluten proteins, predominantly gliadins in wheat, are resistant to complete degradation by mammalian enzymes, which results in the production of large peptides with immunogenic sequences, such as the 33-mer in α-gliadin. Overall, this specific peptide contains 6 copies of 3 different epitopes (PYPQPQLPY, PQPQLYPQ, PFPPQPQLPY) to which most celiac patients react.[3,4] Partially digested gluten peptides translocate the mucosal barrier and are deamidated by human transglutaminase 2 (TG2), the CD-associated autoantigen.[5] This process converts glutamine residues to glutamate and increases peptide binding affinity to HLA-DQ2 or DQ8 heterodimers in antigen-presenting cells, initiating the T-cell–mediated inflammation characteristic of CD.[6] Up to 40% of most populations express the susceptibility genes for CD; however, only 2%–4% will develop disease, possibly due to additional unknown environmental triggers.[7] As with other autoimmune and inflammatory diseases, intestinal dysbiosis characterized by abundance of Proteobacteria and decreases in *Lactobacillus* has been described in some CD patients.[8–10] There is little mechanistic insight regarding the association between dysbiosis and gluten-specific T-cell responses, and the functional relevance of these associations in CD remain unclear.

The human gastrointestinal tract is colonized by bacteria with in vitro gluten-degrading capacity.[11,12] This has prompted the hypothesis that bacteria could reduce gluten immunogenicity by producing enzymes that effectively cleave proteolytic-resistant sequences in gluten peptides.[13] Here we show a complex scenario in which gluten

Abbreviations used in this paper: ASF, altered Schaedler flora; CD, celiac disease; CFU, colony-forming unit; LC-MS/MS, liquid chromatography tandem mass spectrometry; PT, pepsin-trypsin; PBMC, peripheral blood mononuclear cell; SFU, spot-forming units; TG, transglutaminase.

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metabolism in the small intestine of gnotobiotic mice is differentially affected by opportunistic pathogens and commensal bacteria. We demonstrate that *Pseudomonas aeruginosa*, isolated from the duodenum of CD patients, produces, through its elastase activity, a multitude of peptides that activate gluten-specific T cells in HLA-DQ2.5^{+} CD patients. Conversely, *Lactobacillus* spp from healthy subjects, degrade *P aeruginosa*–modified peptides and decrease their immunogenic potential. We identify a microbe–dietary–host interaction that may modulate autoimmune risk in genetically susceptible persons and that could be targeted to reduce the rising incidence of these conditions.

Materials and Methods

Mice

C57BL/6 germ-free mice were generated by axenic 2-cell embryo transfer technique, as described previously,[14] and maintained in flexible film isolators at the McMaster University Axenic Gnotobiotic Unit. Germ-free status was evaluated weekly by a combination of culture and culture-independent techniques.[14,15] We used mice colonized with an 8 strain-murine microbiota (altered Schaedler flora [ASF])[16] as controls. All mice had unlimited access to a gluten-free autoclaved mouse diet (Harlan, Indianapolis, IN) and water. All experiments were carried out in accordance with the McMaster University animal utilization protocols.

Origin of Bacterial Strains Used

We previously sequenced and isolated a collection of bacterial strains with in vitro gluten-degrading capacity from the small intestine of CD patients and non-celiac controls.[17,18] Briefly, duodenal biopsies were incubated in specific gluten media (MCG-3)[11] for 48 hours under anoxic and microaerophilic conditions. Bacteria were selected based on production of a proteolytic halo and lack of growth in the same media without gluten. Most of the strains were classified within the phylum *Firmicutes* (88%), mainly from the genera *Lactobacillus*. Strains were also classified into *Actinobacteria* (8%), *Proteobacteria* (3%), and *Bacteroidetes* (1%).[17] For the experiments in this study, 3 bacterial groups of interest were chosen (Supplementary Table 1). *P aeruginosa* X-46.1 was selected as an opportunistic pathogen only isolated from CD patients,[17] and a member of Proteobacteria, a group previously associated with CD.[8–10] *Staphylococcus* spp was selected because alterations in this group have been described in CD patients.[19] *Lactobacillus* spp from healthy subjects were selected because it constitutes a core resident group in the human small intestine[18,20] that is involved in gluten metabolism in vitro[11] and is altered in CD patients.[21,22]

16S Sequencing

DNA was extracted from small intestinal samples of colonized mice as described previously.[23] Extracted DNA underwent amplification for the hypervariable 16S ribosomal RNA gene v3 region and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA). Generated data were analyzed as described previously. Briefly, sequences were trimmed using Cutadapt software, version 1.2.1, aligned using PANDAseq software, version 2.8, operational taxonomic units selected via AbundantOTU, and taxonomy assigned against the Greengenes reference database.[24,25]

QPQLPY-Peptide Quantification

The amount of QPQLPY-peptide, a key motif in the major immunogenic epitope within the 33-mer peptide from α-gliadin, was measured with the competitive G12 ELISA GlutenTox Kit (Biomedal, Spain) according to the manufacturer's instructions.[26] For animal studies, total small intestinal content was flushed at sacrifice with 3 mL extraction solution provided by the kit.

Degradation of QPQLPY Peptides by Intestinal Washes

Intestinal contents were collected from colonized mice at sacrifice and diluted 1:5 with phosphate-buffered saline and incubated at 37°C with 7 mg pepsin-trypsin (PT)-gliadin for 30 minutes, 2 hours, and 4 hours. After incubations, remaining QPQLPY-peptides were quantified by G12 antibody in ELISA GlutenTox Kit.[26]

Cleavage of Gluten-Derived Tripeptides

Peptidase activity against gluten-derived tripeptides was performed as described previously.[27] Five synthetic analogs—Z-YPQ-pNA, Z-QQP-pNA, Z-PPF-pNA, Z-PFP-pNA, and Z-QPQ-pNa—were chosen as representative gliadin-derived substrates (Biomatik). Twenty millimolars of each peptide was incubated with the small intestinal washes of *P aeruginosa*-, *Lactobacillus* spp- or *Staphylococcus* spp-colonized mice, or with single bacteria cell cultures at the same concentration found in the small intestine of mice (10^4 colony-forming units [CFU]) in 50 mM ammonium bicarbonate buffer (pH 8.0). Enzyme activity was determined by the proteolytic removal of the para-nitroanilide group, which was monitored spectrophotometrically at 405 nm.

Proteolytic Activity in Gluten Media

Degradation of gluten proteins in solid media was measured using bioassays on agar plates containing 1% gluten.[28] Small intestinal contents of mice were diluted 1:5 with phosphate-buffered saline and incubated at 37°C in gluten-agar media for 24 hours. Plates were evaluated by measuring the diameter of the halo formed. Trypsin diluted in saline was used for construction of a standard curve.

Liquid Chromatography Tandem Mass Spectrometry Analysis of 33-Mer–Derived Peptides

Degradation of 33-mer peptide was performed using liquid chromatography tandem mass spectrometry (LC-MS/MS). The reaction mixtures (100 μL) containing 10 μL bacterial culture (10^4 CFU) and 60 μM of the 33-mer peptide in phosphate-buffered saline (pH 7.3), were incubated at 37°C for 4 hours. Reactions were stopped by incubation at 100°C for 10 minutes, and resultant products subjected to LC-MS/MS. LC-MS/MS data were collected using a Bruker AmazonX ion trap mass

spectrometer coupled with a Dionex UltiMate 3000 HPLC system, equipped with a Luna C18 column (150 mm × 4.6 mm; Phenomenex, Torrance, CA) for analytical separations, running acetonitrile with 0.1% formic acid and ddH_2O with 0.1% formic acid as the mobile phase at a flow rate of 1.2 mL/min. Putative gliadin peptides were identified by comparison of LC-MS/MS chromatograms from samples with gliadin to gliadin-free controls. Peptides were confirmed and annotated by manual MS/MS sequencing, assisted by iSNAP LC-MS/MS peptide fragmentation analysis software.[29]

Gluten Challenge in Crohn's Disease Patients

Patients with biopsy-proven CD (n = 20) expressing the most common susceptibility genotype HLA DQ2.5+ and in clinical, serologic, and histologic remission on a gluten-free diet, were recruited (Supplementary Table 2). Gluten challenge was performed as described previously.[30] Briefly, four 50-g slices of standard gluten-containing wheat bread (total approximately 10 g gluten) were consumed in divided doses daily for 3 days. Blood sampling was done in the morning before (day 0) and 6 days after commencing the gluten challenge (day 6).

ELISpot Assay

Interferon-gamma ELISpot assays (Mabtech, Cincinnati, OH) were performed as described previously.[30] Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood using Ficoll density-gradient centrifugation. Fresh or cryopreserved PBMC were incubated overnight with or without native or deamidated gliadin, peptides, or with tetanus toxoid (CSL, Melbourne, Australia) and phytohemagglutinin-L (Sigma-Aldrich, St. Louis, MO) as positive controls. Spot-forming units (SFU) in individual wells were counted using an automated ELISPOT reader. Wells showing >10 SFU and >3× the SFU counted in wells containing PBMCs incubated with medium alone were regarded as positive. SFU were adjusted to 1 million PBMCs plated to enable comparisons.

Peptide Translocation in Mice

Permeability studies were assayed in vitro by Ussing chamber technique, as described previously[31] (World Precision Instruments, Sarasota, FL). We collected jejunal tissues from specific pathogen-free C57BL/6 mice (n = 10 per group), and 4 sections of jejunum from each mouse were assessed. Intestinal permeability was stimulated by adding prostaglandin E2 (30 μM) to the serosal side of the chamber. Tissue conductance and mucosal-to-serosal flux of the paracellular probe ^{51}Cr-EDTA were determined to check integrity of the tissue (data not shown). PT-gliadin (1 mg/mL) and PT-gliadin further degraded by bacteria (10^4 CFU 37°C for 4 hours) were added to the mucosal size of the chamber. After 2 hours, samples were collected from the serosal side of the chamber and gluten content was quantified using the G12 antibody.[26]

Deamidation by Transglutaminase

The enzymatic activity of TG was checked by cross-linking PT-gliadin or PT-gliadin incubated with *P aeruginosa* X-46.1 for 4 hours (glutamine donors) with monodansyl cadaverine (glutamine acceptor). One microgram crude gliadin (Sigma-Aldrich) was incubated with 30 μmol/mL monodansyl cadaverine (Covalab, Aachen, Germany) and 20 μg/mL pig TG (Sigma-Aldrich) in 100 μL buffer containing 0.1 mol/L Tris·HCl, 0.15 mol/L NaCl, and 5 mmol/L $CaCl_2$ (pH 8.8). Cross-linking was allowed for 2 hours at 37°C. Fluorescence was measured for 1 hour at λex 360 nm and λem 535 nm in the kinetic mode.[32]

Screening of the Non-Redundant Transposon Mutant Library of Pseudomonas aeruginosa

An unbiased genetic strategy to identify genes associated with gluten metabolism was performed by using the available non-redundant transposon mutant library of *P aeruginosa* PA14.[33] Briefly, 96-well microtiter plates containing 100 μL per well of LB (with gentamicin at 15 μg/mL) were inoculated directly from each plate of the frozen library using a 96-pin replicator and incubated statically at 37°C overnight. Overnight cultures were then transferred with a 96-pin replicator (VP408; V&P Scientific, San Diego, CA) onto gluten agar plates (1% of gluten) and incubated for 16 hours at 37°C. Transposon mutants with growth on gluten-containing agar and no zone of clearing around spotted colonies were used as stringent selection criteria and reported in Supplementary Table 3.

Statistics

All the variables were analyzed with SPSS, version 18.0 (SPSS Inc, Chicago, IL). Categorical variables are expressed as numbers and percentages, and quantitative variables as means ± SEM or medians as appropriate. Data are depicted as either dot plots or bar graphs. The analysis of variance test was performed to evaluate differences between various samples with a parametric distribution and a Bonferroni correction was applied. The Student *t* test was performed to evaluate the differences between 2 independent samples or paired samples as appropriate. Data with nonparametric distribution were evaluated with Kruskal-Wallis test for multiple samples, Mann-Whitney test for 2 independent samples or Wilcoxon test for 2 related samples as appropriate. A *P* value <.05 was selected to reject the null hypothesis by 2-tailed tests.

Results

Commensals and Opportunistic Pathogens Contribute to Gluten Metabolism in the Gut

To investigate the small intestinal gluten metabolic activity of the strains selected in this study, we colonized germ-free C57BL/6 mice (n = 13/group) with *P aeruginosa* X-46.1, a proteobacteria isolated from the duodenum of CD patients; *Staphylococcus epidermidis* X-35.1 and *Staphylococcus warneri* X-18.3 from the duodenum of CD patients; and *Lactobacillus rhamnosus* X-32.2 and *Lactobacillus fermentum* X-39.3 from the duodenum of non-celiac healthy volunteers (Supplementary Table 1). Mice were colonized by oral gavage with 10^7 CFU of each strain and kept on gluten-free chow for 1 week. Control groups included germ-free mice and ASF-colonized mice, a bacterial community selected for their dominance and persistence in the normal microbiota of mice[16] (Figure 1*A*). 16S sequencing of ASF small-intestinal contents showed 90%

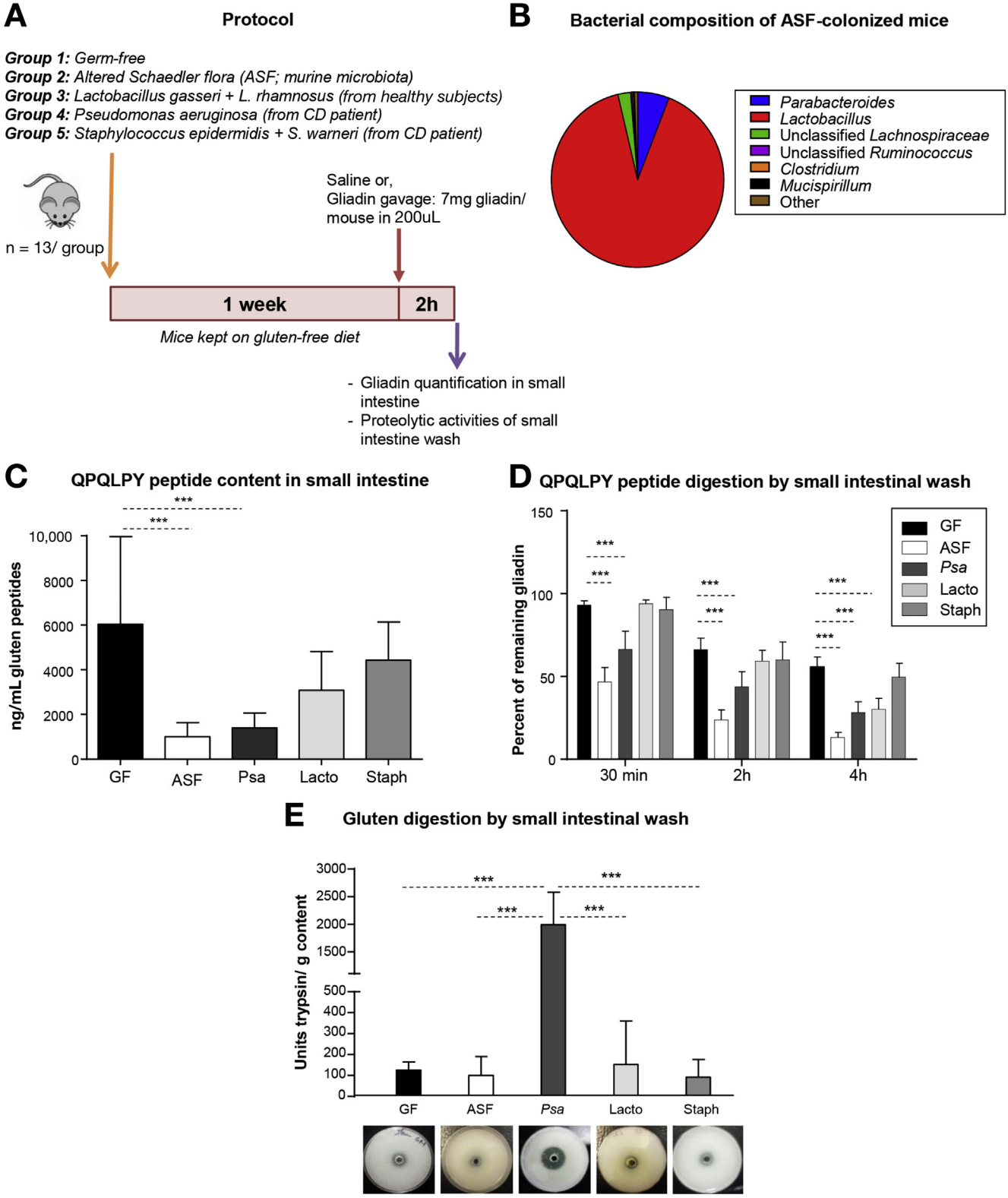

Figure 1. Resident intestinal bacteria participate in gliadin degradation. (*A*) Experimental design of mouse colonization study. Q16 (*B*) 16S sequencing of small-intestinal bacteria in ASF-colonized mice at the genus level. (*C*) Amount of gliadin measured by G12 antibody, detecting the immunogenic QPQLPY sequence within the 33-mer, in the small intestine of germ-free (GF), ASF-, *P aeruginosa*-, *Lactobacillus* (*Lacto*)-, and *Staphylococcus* (*Staph*)-colonized mice 2 hours after gliadin gavage. Data are represented as mean ± SEM. (*D*) Degradation of 7 mg PT-gliadin by intestinal washes from germ-free, ASF-, *P aeruginosa*, *Lacto*-, and *Staph*-colonized mice by measuring QPQLPY peptides by G12 antibody. Data are represented as mean ± SEM. (*E*) Degradation of gluten in solid media by intestinal washes of GF, ASF-, *P aeruginosa*-, *Lacto*-, and *Staph*-colonized. Data represented as mean ± SEM (units of trypsin/gram of intestinal content). Bioassays show nonspecific degradation (clearing zone) in solid gluten media (*white*).

Lactobacillus, 5% *Parabacteroides*, and other minority groups (Figure 1*B*). One-week post-colonization, mice were gavaged with 7 mg gliadin (n = 8/group) or with saline (n = 5/group), and sacrificed after 2 hours. We recovered 10^4–10^5 CFU/g of intestinal content in each group. Culture analysis[14] confirmed the purity of colonizations (Supplementary Materials). The content of QPQLPY peptides, a repetitive immunogenic sequence within the 33-mer peptide, was quantified by the G12 antibody.[26] Colonization with ASF or *P aeruginosa* decreased QPQLPY peptides content compared to germ-free mice, which exhibited a range of values reaching a maximum of 12,000 ng/mL (Figure 1*C*).

To investigate whether these differences were due to bacterial proteolytic activity, intestinal washes from colonized mice were incubated with 7 mg partially degraded, immunologically active PT-gliadin for 30 minutes, 2 hours, and 4 hours. Small intestinal washes from ASF-colonized mice degraded 50%–60% of QPQLPY-peptides within 30 minutes, while washes from germ-free mice degraded only 5%–10%. Intestinal washes from *P aeruginosa*–colonized mice degraded >50% of peptides at 2 hours and washes from *Lactobacillus*-colonized mice reached similar activity at 4 hours (Figure 1*D*). Intestinal washes from *Staphylococcus*-colonized mice also degraded QPQLPY peptides, but this did not reach statistical significance compared with germ-free mice. Because gluten consists of a complex mix of proteins with multiple amino-acid sequences, we next tested the ability of small-intestinal washes to degrade whole gluten. Unlike the G12 antibody that detects the QPQLPY sequence, the bioassay assesses global gluten degradation non-specifically. Using solid gluten media, we showed that intestinal washes from *P aeruginosa*–colonized mice had 10× higher proteolytic activity than the rest of the groups (Figure 1*E*). Thus, gluten degradation in the small intestine results from the combined enzymatic action of mammalian and resident bacteria.

Small Intestinal Bacteria Induce Distinct Gluten Metabolic Patterns

To investigate specific regions of gliadin cleavage by bacteria, 5 tripeptides, representing sequences that appear frequently in immunogenic gliadin peptides, were incubated with intestinal washes of colonized mice. Single bacterial strains that were used in colonizations were directly incubated in vitro with the 5 tripeptides as controls. Intestinal washes of all groups, including germ-free, cleaved YPQ and PPF (Figure 2*A*), suggesting mammalian origin. Both *P aeruginosa* X-46.1 and *Staphylococcus* strains degraded PPF tripeptide and mice colonized with these bacteria showed higher PPF degradation than germ-free mice. Thus, different bacteria could have similar degradation profiles that are conferred to mice colonized with these strains. Furthermore, direct incubation of *L rhamnosus* X-32.2 with tripeptides resulted in PFP breakdown. Similarly, incubation of intestinal washes of *Lactobacillus*- and ASF-colonized mice, which are dominated by *Lactobacillus*, resulted in PFP breakdown. Intestinal washes of ASF-colonized mice demonstrated PQP cleavage, not present in germ-free mice. The results suggest there are specific bacterial cleavage sites that influence the pool of gliadin peptides produced during digestion.

To identify the capacity of specific human bacterial isolates to cleave key immunogenic gliadin peptides, we incubated bacteria with the human protease resistant 33-mer peptide that encompasses 6 overlapping immunodominant HLA-DQ2.5–restricted 9-mer T-cell epitopes.[3,6] Peptides generated from the 33-mer after incubation with bacteria were determined using LC-MS/MS. Partial scission of the 33-mer was detected with all tested bacteria. In isolation, *Lactobacillus* spp produced 3 peptides of 25–32 amino acids and *Staphylococcus* X-18.3 two large peptides of 28 and 32 amino acids. *P aeruginosa* X-46.1 cleaved regions recognized by G12 antibody and produced a variety of smaller 33-mer derived peptides (10–30 amino acids). *P aeruginosa* X-46.1 did not cleave QLP regions in the 33-mer, which are associated with immunogenicity[6] (Figure 2*B* and *C*). These results indicate that degradation of the 33-mer by bacteria generate gluten peptides that maintain sequences with known immunogenicity in CD.[30]

Pseudomonas Aeruginosa-*Modified Gluten Peptides Are Immunogenic to Crohn's Disease Patients*

The immunogenicity of 33-mer–derived peptides released by *P aeruginosa* X-46.1 was then tested using gluten-specific T cells induced in HLA-DQ2.5$^+$ CD patients. Ten CD patients underwent 3-day wheat gluten challenge to induce gluten-specific T cells[30,34] (Supplementary Table 2). PBMCs were isolated from blood collected before and 6 days after commencing the challenge, when circulating gluten-specific T cells are at their peak. A panel of 4 gluten peptides generated after 33-mer incubation with *P aeruginosa* X-46.1 were synthesized. Interferon gamma ELISpot using these PBMCs was performed to validate the immunogenicity of these peptides, as well as PT-gliadin and the 33-mer peptide. Seven of 10 participants mounted a significant interferon-gamma ELISpot response on day 6 to the 33-mer, PT-gliadin, and all 4 *P aeruginosa* peptides (Figure 3*A*). Responses were detected only after gluten challenge and were generally dose-dependent and enhanced by deamidation, consistent with a disease-relevant T-cell response to deamidated gluten (Supplementary Figure 1).

We next assessed the immunogenicity of PT-gliadin incubated with either *P aeruginosa* X-46.1 or *Lactobacillus* spp. Gluten-specific T-cell responses to these peptides were performed using blood collected from CD patients (Supplementary Figure 2). The median response to PT-gliadin incubated with *P aeruginosa* X-46.1 was increased compared with deamidated PT-gliadin alone, and this was statistically significant after 8-hour incubation with *P aeruginosa* X-46.1. In contrast, the median response to PT-gliadin incubated with *Lactobacillus* spp was lower than that to deamidated PT-gliadin at all incubation time points (Figure 3*B*).

A Cleavage of gluten-derived tripeptides by bacteria and small intestinal washes

	GF	ASF	*Pseudomonas*		*Lactobacillus spp.*			*Staphylococcus spp.*		
	SIW	SIW	*P. aeruginosa*	SIW	*L. rhamnosus*	*L. gasseri*	SIW	*S. epidermidis*	*S. warneri*	SIW
YPQ	+	+	-	+	-	-	+	-	-	+
QQP	-	-	-	-	-	-	-	-	-	-
PPF	+	+	++	++	-	-	+	+	++	++
PFP	-	+	-	-	+	-	+	-	-	-
PQP	-	+/-	-	-	-	-	-	-	-	-

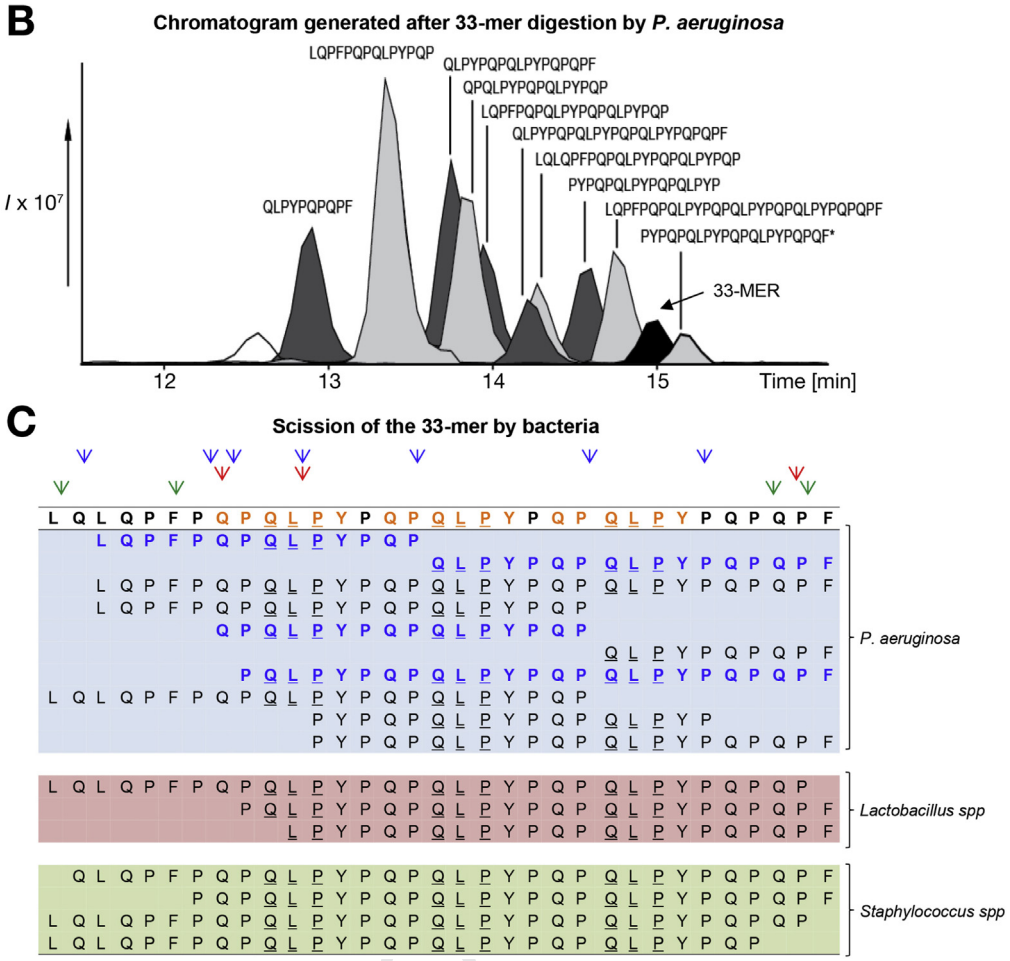

Figure 2. Intestinal bacteria induce distinct gluten metabolic patterns against gliadin peptides. (*A*) Cleavage of gluten-derived tripeptides YPQ, QQP, PPF, PFP, PQP by small intestinal washes (SIW) of germ-free (GF)-, ASF-, *P aeruginosa*-, *Lactobacillus*-, *Staphylococcus*-colonized mice and by individual bacterial strains. No activity:(−), activity:(+), saturated activity:(++). (*B*) Chromatogram generated after 33-mer degradation by *P aeruginosa*. *Black peak*: remaining 33-mer. *Gray peak*: peptides produced by *P aeruginosa* degradation. (*C*) Degradation of the 33-mer by *P aeruginosa* (*blue arrows*), *Lactobacillus* (*red arrows*), and *Staphylococcus* (*green arrows*). *Yellow letters*: G12 antibody epitopes. *Underlined*: TG2 epitopes. *Blue letters*: Selected peptides for gluten-specific T-cell stimulation assays (*$P < .05$; ***$P < .005$).

Pseudomonas Aeruginosa*-Modified Gluten Peptides Translocate Better the Epithelial Barrier in Mice*

Uptake of gluten peptides through the intestinal barrier is necessary for the adaptive gluten-specific immune response in the lamina propria.[35] We measured translocation of immunogenic gluten peptides in mouse jejunum by Ussing chambers.[31] PT-gliadin or PT-gliadin incubated with *P aeruginosa* X-46.1 was added to the mucosal side of the chamber and QPQLPY peptide content, as measured by G12 antibody, was determined on the serosal side after 2 hours.[26] Prior incubation of PT-gliadin with *P aeruginosa* X-46.1 led to increased QPQLPY peptide transport across the intestinal barrier, compared with PT-gliadin alone (Figure 3*C*). This suggests that *P aeruginosa*–modified gliadin peptides crossed the mucosal barrier more efficiently and that bacterially mediated gliadin degradation in the lumen may facilitate immunogenic peptide translocation (Figure 3*C*). Gliadin peptide deamidation in the lamina propria by the CD autoantigen TG2 increases peptide affinity to HLA-DQ2^{+} antigen-presenting cells.[5] Different ratios of deamidation after bacterial degradation could therefore be associated with reduced immunogenicity. We found a similar TG2 deamidation ratio of PT-gliadin and PT-gliadin incubated with *P aeruginosa* X-46.1 (Figure 3*D*). Thus, *P aeruginosa* degradation produced shorter gluten peptides that were often highly immunogenic, inducing responses in many cases as strong as the parent 33mer. These shorter peptides readily crossed the epithelial barrier.

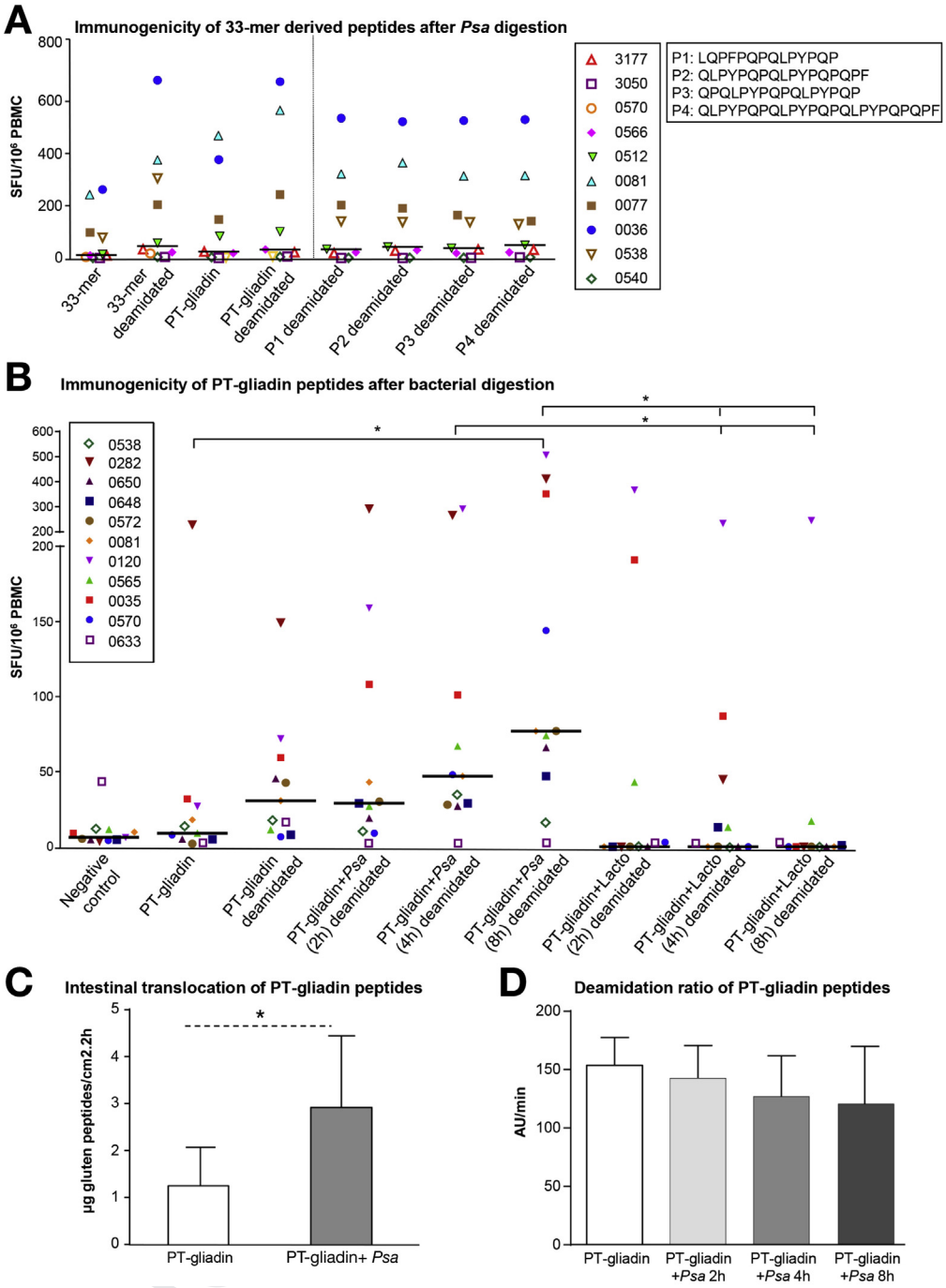

Figure 3. Intestinal bacteria modify the immunogenicity of gluten peptides. (*A*, *B*) Interferon-gamma ELISpot in PBMCs harvested after gluten challenge of patients with CD in remission and ex vivo stimulated with (*A*) TG2-deamidated 33-mer-derived peptides (P1, P2, P3, P4) produced after incubation with *P aeruginosa* X-46.1 or (*B*) pepsin-trypsin predigested (PT) gliadin incubated with *P aeruginosa* X-46.1 or *Lactobacillus* and deamidated by TG2. Non-deamidated and TG2-deamidated 33-mer or PT-gliadin were used as controls. Results are shown as SFU per 10^6 PBMC. Median response is represented by *horizontal lines*. Each patient donor response (numeric code) is represented with *characteristic shape* and *color dots*. (*C*) Small intestinal translocation of PT-gliadin and PT-gliadin incubated with *P aeruginosa* X-46.1 as measured by Ussing chambers. Results shown as the transport of QPQLPY gliadin peptides from the mucosal to the serosal side over 2 hours. Data are represented as mean ± SEM. (*D*) Cross-linking of PT-gliadin and PT-gliadin incubated with *P aeruginosa* for 2, 4, and 8 hours to monodansyl cadaverine by TG2. Results are shown as maximum rates of cross-linking to monodansyl cadaverine (AU/min) (*$P < .05$; ***$P < .005$).

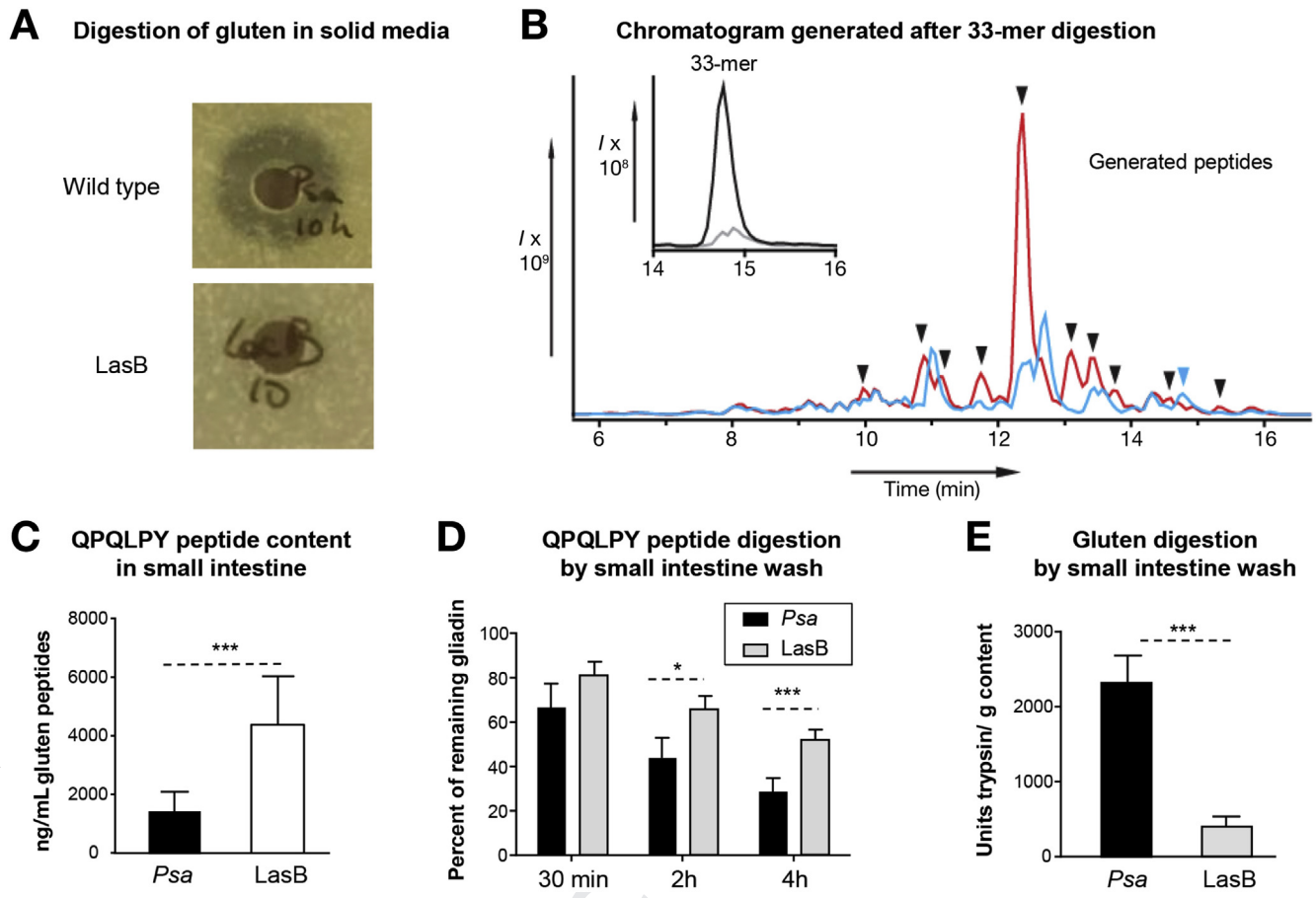

Figure 4. *LasB* elastase is involved in gluten degradation by *P aeruginosa*. (*A*) Degradation of gluten proteins in solid media of *P aeruginosa* PA14 wild-type and *LasB* mutant. Bioassays show nonspecific degradation (clearing zone) in solid gluten media (*white*). (*B*) Cleavage of the 33-mer peptide (*blue arrow*) by *P aeruginosa* PA14 (*red chromatogram*) and *LasB* mutant (*blue chromatogram*). *Black arrows*: peptides generated after 33-mer degradation by *P aeruginosa* PA14. *Inset* shows 33-mer after *LasB* mutant incubation (*black chromatogram*) and after *P aeruginosa* PA14 incubation (*gray chromatogram*). (*C*) Germ-free mice were colonized with *P aeruginosa* or LasB mutant for 1 week, after which they were gavaged with 7 mg gliadin. The amount of the QPQLPY sequence in the small intestine of colonized mice was measured by G12 antibody 2 hours after gliadin gavage. Data are represented as mean ± SEM. (*D*) Degradation of 7 mg pepsin-trypsin predigested (PT)-gliadin by intestinal washes from *P aeruginosa*–colonized mice and LasB-colonized mice after 30 minutes, 2 hours, and 4 hours incubation. The amount of the QPQLPY peptides after incubation was measured by G12 antibody. Data are represented as mean ± SEM. (*E*) Degradation of nonspecific gluten proteins in solid media by intestinal washes of *P aeruginosa*–colonized mice and *LasB*-colonized mice. *Bar graph* shows units of trypsin per gram of intestinal washes based on a standard curve with trypsin. Data represented as mean ± SEM (*$P < .05$; ***$P < .005$).

LasB *Elastase Is the Main Protease Involved in Gluten Metabolism by* Pseudomonas aeruginosa

We first confirmed that *P aeruginosa* PA14, a human clinical isolate,[36] had an identical degradation pattern to *P aeruginosa* X-46.1 against gluten and 33-mer (data not shown). To identify genes associated with gluten metabolism, we used the available non-redundant transposon mutant library of PA14.[33] Approximately 6000 transposon mutants were tested for their ability to degrade gluten and 23 mutants consistently failed to generate a typical hydrolytic halo surrounding colonies on gluten-containing agar (Supplementary Table 3). These mutants included *LasB*, which encodes elastase, genes involved in the expression of *LasB* elastase, and the type II system known for the secretion of exoenzymes including *LasB* in *P aeruginosa*.[37] Our analysis supports that *LasB* was the main extracellular protease involved in gluten degradation, and consistent with these results, a *LasB* mutant had no peptidase activity against the 33-mer compared with its wild-type parent strain PA14 (Figure 4*A* and *B*). Colonization of germ-free mice with this mutant showed a reduction of gluten and gliadin degradation, and an increase of gliadin QPQLPY peptides in the small intestine compared with *P aeruginosa* X-46.1-colonized mice (Figure 4*C*–*E*), further supporting its role in gluten metabolism in vivo.

Immunogenic Peptides Produced by Pseudomonas aeruginosa *Are Detoxified by* Lactobacillus *spp*

The intestinal microbiota is a dynamic community where bacteria coexist with the host and with other bacteria.[38]

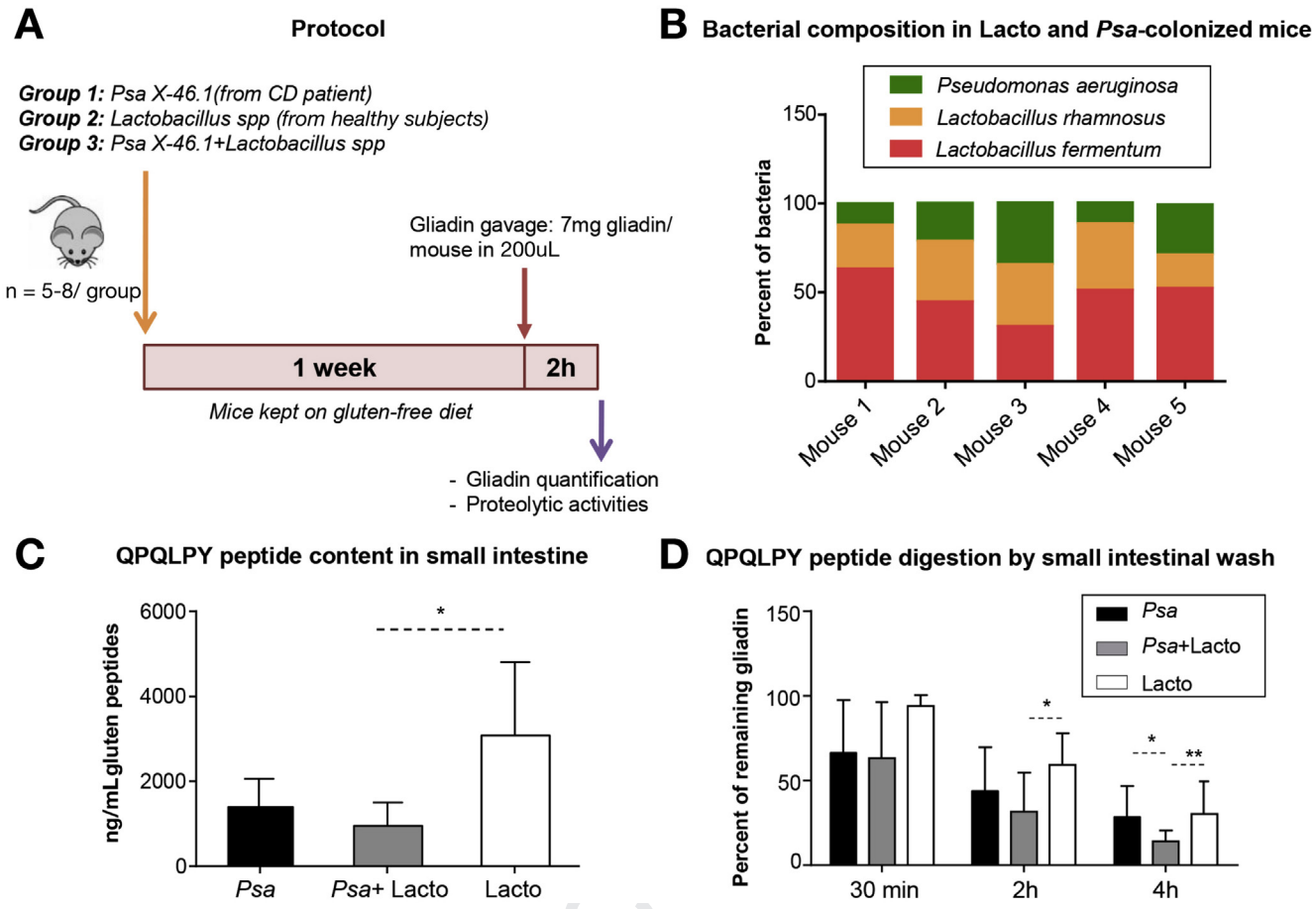

Figure 5. Peptides modified by *P aeruginosa* can be degraded by *Lactobacillus* (Lacto). (*A*) Experimental design of mouse colonization study. (*B*) Bacterial composition in the small intestine of mice colonized with *Lactobacillus* spp and *P aeruginosa*. (*C*) Amount of QPQLPY-peptides measured by G12 antibody in the small intestine of *P aeruginosa*-, *Lactobacillus*- (*Lacto*-), and *P aeruginosa*+*Lactobacillus*-colonized mice 2 hours after gliadin gavage (7 mg). Data are represented as mean ± SEM. (*D*) Degradation of 7 mg of pepsin-trypsin predigested (PT)-gliadin by intestinal washes from *P aeruginosa*-, *Lacto*-, and *P aeruginosa*+*Lacto*-colonized mice by measuring QPQLPY peptides. Data are represented as mean ± SEM (*$P < .05$; ***$P < .005$).

Combinations of bacteria producing different proteases could further affect gliadin degradation products. To investigate this, we colonized C57BL/6 mice with *P aeruginosa* X-46.1 and *Lactobacillus* spp. Controls were colonized with *P aeruginosa* X-46.1 or with *Lactobacillus* spp. One week post-colonization, mice were gavaged with 7 mg gliadin and QPQLPY peptide content was measured in the small intestine after 2 hours (Figure 5*A*). Mice were successfully colonized with *P aeruginosa*+*Lactobacillus* spp (Figure 5*B*) and they had lower QPQLPY gliadin peptide content compared with mice colonized with *Lactobacillus* alone (Figure 5*C*). Incubation of intestinal washes from colonized mice with PT-gliadin demonstrated that the combination of *P aeruginosa* X-46.1 and *Lactobacillus* spp enhanced QPQLPY degradation (70% in 2 hours) compared with *P aeruginosa*- (55%) and *Lactobacillus*-colonized mice (40%) (Figure 5*D*). We then analyzed whether *Lactobacillus* reduced major immunogenic peptides generated by *P aeruginosa* X-46.1. We sequenced the peptides produced by *P aeruginosa* X-46.1. These peptides were then incubated with *Lactobacillus* spp and analyzed by LC-MS/MS. Compared with *Lactobacillus* spp-mediated degradation of intact 33-mer, *Lactobacillus* spp degraded *P aeruginosa*−modified 33-mer derived peptides more efficiently, delivering peptides of 4−12 amino acids (Figure 6*A*). Most of these peptides are shorter than the 9 amino acids required for efficient antigen binding to HLA-DQ2 and activation of T cells. We confirmed this by measuring gluten-specific T-cell responses to these peptides using CD patients who underwent wheat challenge (Supplementary Table 2 and Supplementary Figure 3).[34] Only a minority of *Lactobacillus* spp degraded *P aeruginosa*−modified 33-mer−derived peptides showed immunogenicity and, overall, there was a reduction of immunogenicity compared with *P aeruginosa*−modified peptides (Figure 6*B*). The results suggest immunogenic peptides generated by *P aeruginosa* can be degraded to non-immunogenic peptides in the presence of *Lactobacillus* spp.

Discussion

The role of intestinal microbiota in health and disease has been one of most studied areas in the past decade,[39] and its contribution to food sensitivities[40] and autoimmune

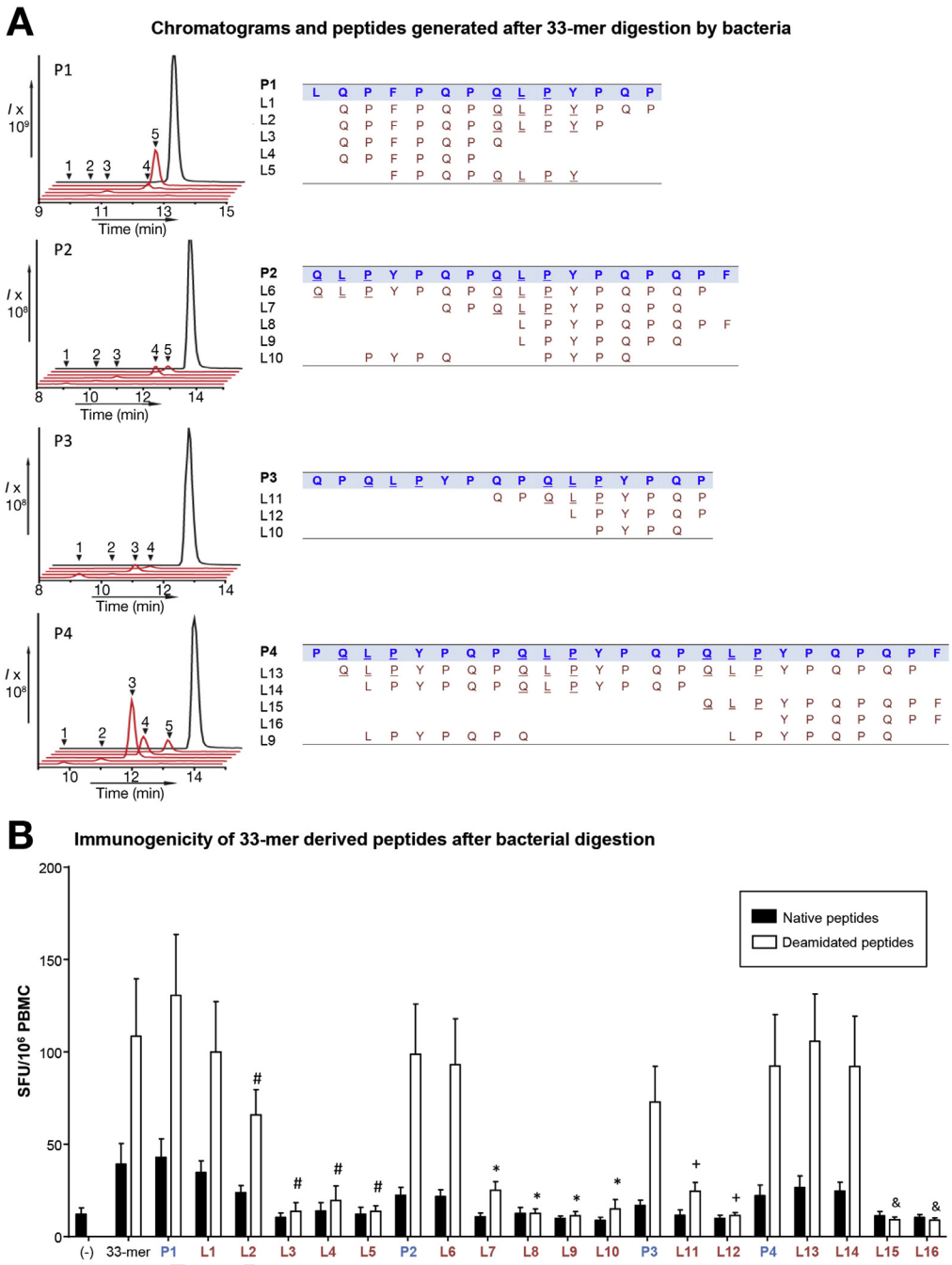

Figure 6. Immunogenic peptides produced by *P aeruginosa* can be degraded to non-immunogenic peptides by *Lactobacillus* spp. (*A*) Chromatograms and sequences of peptides generated after 33-mer degradation by *P aeruginosa* (P1−P4) that were further degraded by *Lactobacillus* (L1−L16). Chromatograms (*left*) show *P aeruginosa*−derived 33-mer peptides (P1−P4; *black peaks*) generated by *Lactobacillus* degradation (*red peaks*). Tables (*right*) show sequences of *P aeruginosa*−derived 33-mer peptides (P1−P4; in *blue*) and peptides produced by *Lactobacillus* from *P aeruginosa*−derived 33-mer peptides (L1−L16; in *red*). *Underlined*: TG2-epitopes. (B) Interferon-gamma ELISpot in PBMCs harvested after gluten challenge in CD patients in remission and ex vivo stimulated with native and deamidated peptides produced by Lacto (L1−L16) from *P aeruginosa*−derived 33-mer peptides (P1−P4). TG2-deamidated and non-deamidated 33-mer were used as controls. Results are shown as SFUs per 10^6 PBMC. Median response represented by *horizontal line*. Each patient donor response (numeric code) is represented with *characteristic shape* and *color dots* ($^{\#}P < .05$ vs deamidated P-1; $^{*}P < .05$ vs deamidated P-2; $^{+}P < .05$ vs deamidated P-3; $^{\&}P < .05$ vs deamidated P-4 peptide).

disorders, such as CD,[41] is emerging. CD represents a unique model to study diet-induced intestinal inflammation and autoimmunity because the main environmental trigger, gluten, has been identified as well as the molecular mechanisms underlying peptide association with MHC class II and subsequent T-cell activation.[6] Here we demonstrate

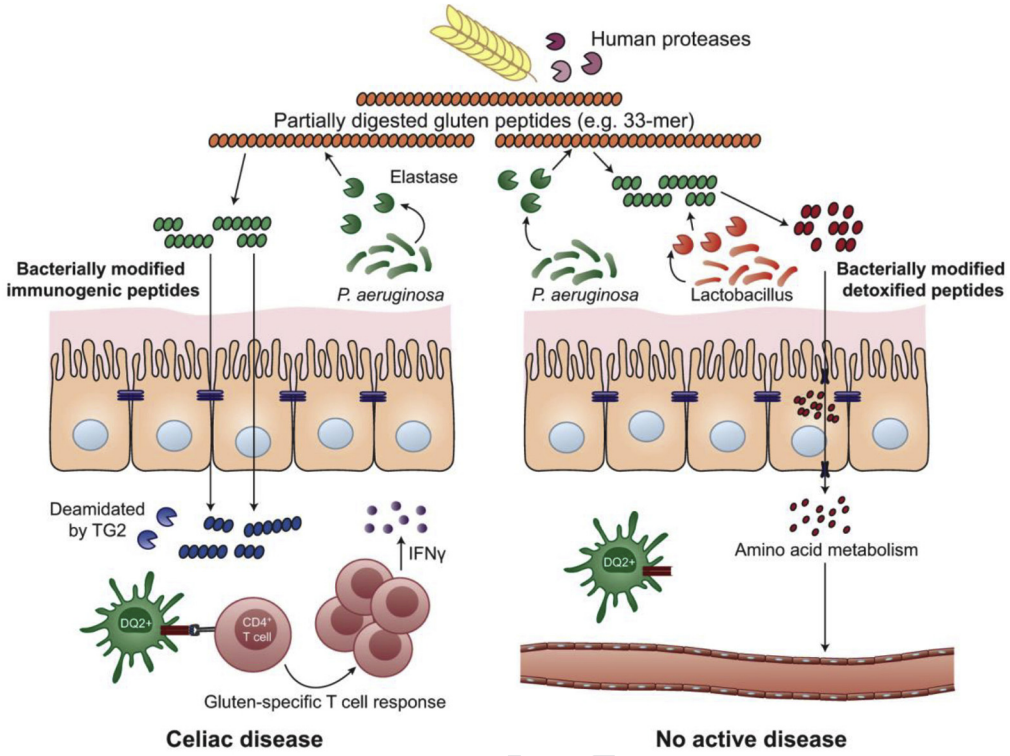

Figure 7. Model depicting how imbalances between pathobionts and core commensals in the proximal small intestine could affect susceptibility to CD in a genetically predisposed host. Gluten proteins rich in proline residues are only partially digested by human proteases generating large and immunogenic peptides, such as the 33-mer peptide. Partially degraded gluten peptides (eg, 33-mer) could be metabolized by opportunistic pathogens, such as *P aeruginosa*, producing slightly shorter peptides, but with retained immunogenicity. These *P aeruginosa*–modified gluten peptides translocate more efficiently the mucosal barrier to interact with antigen-presenting cells expressing HLA-DQ2. On the other hand, *P aeruginosa*–modified gluten peptides can be further detoxified by other members of the duodenal microbiota, such as *Lactobacillus* spp. The metabolic activity of *Lactobacillus* produces peptides shorter than the 9 amino-acid length required for efficient antigen binding to HLA-DQ2 and activation of T cells.

that bacteria from the human small intestine participate in gluten metabolism, and we characterize the pool of peptides produced during bacterial gluten degradation. We show that the tested opportunistic pathogens and core gut commensals generate distinct breakdown patterns of gluten with increased or decreased immunogenicity that could influence autoimmune risk.

The Western diet contains about 20 g gluten per day.[42] Gluten proteins are resistant to mammalian protease degradation, but are good substrates for bacterial metabolic activity.[11] The use of proteases produced by environmental microorganisms have been proposed as pharmacologic therapy in CD.[43–45] However, the ability of these proteases to effectively degrade the amount of gluten present in a normal diet before reaching the small intestine has been questioned.[42] This may limit enzymatic therapy in CD to prevention of gluten-induced effects due to inadvertent gluten consumption in patients who are already on a gluten-free diet. On the other hand, detoxification of gluten in situ by the metabolic activity of resident small intestinal bacteria could constitute an attractive approach. The gluten-degrading capacity of opportunistic pathogens isolated from human feces, such as *P aeruginosa*, has also been recently proposed.[46] We found that *P aeruginosa* cleaves the proteolytic resistant 33-mer gluten peptide, a product of mammalian enzyme degradation, but delivers peptides longer than 9 amino acids that strongly stimulate gluten-specific responses in disease-relevant T cells isolated from HLA-DQ2 CD patients. In addition, incubation of *P aeruginosa* with gliadin predegraded by human proteases enhances peptide immunogenicity in CD patients. The effect on bioactivity is presumed to relate to proteolytic action of the bacteria, but chemical modification via other mechanisms, such as peptide deamidation by *P aeruginosa* transglutaminases[47] could be important. Although our results show a similar deamidation ratio of PT-gliadin with and without bacterial incubation by human TG2, bacterial TGs could deamidate proteins differently. Partial *P aeruginosa* gluten degradation can also facilitate uptake of shorter peptides, but with retained immunogenicity through paracellular or transcellular pathways, increasing their availability to antigen-presenting cells in CD patients. We found that *P aeruginosa*–modified peptides translocate the mucosal barrier more efficiently than peptides generated by human proteases. Finally, through a genomic approach, we identified *LasB*, a metalloprotease virulence factor that could play a pivotal role in infection, as the main bacterial protease involved in the gluten metabolic activity of *P*

aeruginosa. *LasB* has been demonstrated to be important in numerous infection models and it has been a target for the development of anti-pseudomonal therapy.[48] We therefore propose that opportunistic pathogens, such as *P aeruginosa*, colonizing the small intestine may constitute an additional pathogenic factor in CD through their gluten metabolic activity. This highlights the importance of characterizing microbial proteases involved in gluten metabolism, as well as the derived peptides released, before they are proposed as pharmacologic therapy in CD.

Bacterial interactions could affect gluten-degradation patterns and peptide output. In mice, we found that bacterial communities dominated by *Lactobacillus,* such as ASF, showed fast and effective gluten metabolism. In agreement with this, our previous work has shown a protective role of this community in mouse models of gluten sensitivity[15] and *Lactobacillus* have been suggested previously as potential beneficial organisms in CD.[49,50] In isolation, *Lactobacillus* spp do not efficiently degrade the 33-mer. However, incubation of gliadin predegraded by pepsin and trypsin with *Lactobacillus* strains reduces its immunogenicity to gluten-specific T cells from CD patients. This suggests *Lactobacillus* can detoxify gliadin peptides after partial digestion by human proteases. In addition, we found that immunogenic peptides produced by *P aeruginosa* proteases are also further degraded and rendered less immunogenic in the presence of *Lactobacillus*. This mechanism provides an explanation linking imbalances between pathobionts and core commensals, such as *Lactobacillus*, and susceptibility to autoimmune disease in a genetically predisposed host.

It is important to stress that the strains tested in this study are not the only ones that could potentially modify CD risk. Several bacterial groups from the human gastrointestinal tract have been implicated in gluten metabolism in vitro.[11,12] In addition, studies using 16S sequencing continue to identify pathobionts, particularly from protebacteria, present and abundant in populations of CD patients.[10] In addition to modification of gluten immunogenicity, it is possible some pathobionts influence CD risk through nonspecific proinflammatory effects, such as altering intestinal permeability or the innate immune response.

In summary, we identify both pathogenic and protective microbe–gluten–host interactions that may modulate autoimmune risk in HLA-DQ2 susceptible persons. We show that *P aeruginosa* elastase generates highly immunogenic gliadin peptides that translocate through the mucosal barrier. However, *Lactobacillus* further degrade the elastase products to peptides with lower immunogenicity (Figure 7). The mechanisms described in this article could be targeted to reduce disease by inhibiting elastase and similar proteases[51] or increasing the protective enzymatic activity of certain bacteria.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2016.06.041.

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Author contributions: A.C., E.F.V. conceptualized the study, designed experiments, analyzed data, and wrote the manuscript. J.C., A.R.H., and A.C. isolated gluten-degrading bacteria. A.C., H.J.G., and J.L.M. performed germ-free animal studies. J.A.T.-D. and A.K.R. performed gluten-specific T-cell responses. N.A.M. and C.W.J. performed LC-MS/MS analysis. Screening of mutant library was accomplished by S.P.B. and M.G.S. J.J. performed Ussing Chamber experiments. DS contributed to scientific concepts and discussion. All of the authors discussed the results and assisted in the preparation of the manuscript.

Conflicts of interest
This author discloses the following: Jason A. Tye-Din is a co-inventor of patents pertaining to the use of gluten peptides in therapeutics, diagnostics, and non-toxic gluten, and is a shareholders of Nexpep Pty Ltd and a consultant to ImmusanT, Inc. Full disclosure was provided to all study participants. The remaining authors disclose no conflicts.

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