

A Commensal Bifidobacterium longum Strain Prevents Gluten-Related Immunopathology in Mice through Expression of a Serine Protease Inhibitor

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ABSTRACT Microbiota-modulating strategies, including probiotic administration, have been tested for the treatment of chronic gastrointestinal diseases despite limited information regarding their mechanisms of action. We previously demonstrated that patients with active celiac disease have decreased duodenal expression of elafin, a human serine protease inhibitor, and supplementation of elafin by a recombinant Lactococcus lactis strain prevents gliadin-induced immunopathology in the NOD/DQ8 mouse model of gluten sensitivity. The commensal probiotic strain Bifidobacterium longum NCC2705 produces a serine protease inhibitor (SrP) that exhibits immune-modulating properties. Here, we demonstrate that B. longum NCC2705, but not a SrP knockout mutant, attenuates gliadin-induced immunopathology and impacts intestinal microbial composition in NOD/DQ8 mice. Our results highlight the beneficial effects of a serine protease inhibitor produced by commensal B. longum strains.

IMPORTANCE Probiotic therapies have been widely used to treat gastrointestinal disorders with variable success and poor mechanistic insight. Delivery of specific anti-inflammatory molecules has been limited to the use of genetically modified organisms, which has raised some public and regulatory concerns. By examining a specific microbial product naturally expressed by a commensal bacterial strain, we provide insight into a mechanistic basis for the use of B. longum NCC2705 to help treat gluten-related disorders.

KEYWORDS probiotic, microbiota, gluten, serpin, celiac, commensal

Microbiota-modulating therapies have been tested for the treatment of chronic gastrointestinal diseases and disorders with inconsistent findings. Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (57). Specific strains have shown modest efficacy in treatment of irritable bowel syndrome (IBS) (1); of complications of inflammatory bowel disease (IBD), such as pouchitis (2); and of celiac disease (CeD), a chronic enteropathy caused by ingestion of gluten-containing cereals in genetically susceptible individuals (3). In particular, a number of strains belonging to the genus Bifidobacterium have been proposed as beneficial supplements for a wide range of health conditions (4). Depletions in bifidobacteria have been noted in patients with CeD (5), and attempts have been made to supplement some strains as a therapy for CeD (3, 6). However, despite great public interest in the clinical use of specific probiotic strains for intestinal
disorders, there is insufficient mechanistic insight to rationalize consistent recommendations. Investigating therapeutic effects of specific molecules produced by probiotic strains may help bridge this gap.

Dysregulated proteolytic balance has been described in several gastrointestinal disorders (7–11). We have previously shown that expression of the human serine protease inhibitor (serpin) elafin is decreased in the duodenum of patients with active CeD (10, 12). Recombinant Lactobacillus lactis expressing elafin (L. lactis-elafin) has been shown (i) to be protective in several murine colitis models (10) and (ii) to prevent gluten immunopathology in the NOD/DQ8 mouse model of gluten sensitivity (12). However, given the concerns raised with the clinical application of such genetically modified organisms (GMOs), we investigated the effect of a commensal bacterium that naturally expresses an elafin-like serpin. The role of serpins produced by bacteria is unknown, but they are thought to contribute to host-commensal mutualism as these serpins likely provide protection from host proteases (13, 14). Although eukaryotic serpins such as elafin are known to possess anti-inflammatory properties (8), bacterially produced serpins have not been explored for their therapeutic capacity in vivo. The infant-derived commensal probiotic strain Bifidobacterium longum NCC2705 (B. longum srp+) produces a serpin (SrP) encoded by the BL0108 (srp) gene in a nonconstitutive manner. Expression of srp is induced in the murine intestinal tract, and SrP may exhibit anti-inflammatory properties as it inhibits both pancreatic elastase and neutrophilic elastase in vitro (13). We tested the hypothesis that administration of the commensal B. longum srp+ prevents immunopathology in the NOD/DQ8 mouse model of gluten sensitivity.

We show that both the wild-type B. longum srp+ strain and a recombinant strain constitutively expressing srp (B. longum srp+Con) prevent gliadin-induced immunopathology in NOD/DQ8 mice, whereas an srp knockout strain (B. longum Δsrp) does not. These results clearly suggest that the beneficial effect of B. longum srp+ is mediated by SrP. This warrants clinical investigation of commensal B. longum srp+ in managing CeD and nonceliac gluten/wheat sensitivity (NCG/WS) or chronic gastrointestinal conditions associated with proteolytic imbalance.

RESULTS

B. longum srp+ and L. lactis-elafin are equally effective in preventing gliadin immunopathology in mice. We initially compared the efficacy of B. longum srp+ treatment with the efficacy of elafin delivery by recombinant L. lactis, previously shown to prevent intraepithelial lymphocytosis in NOD/DQ8 mice sensitized with gliadin (Fig. 1A) (12). Mice treated with L. lactis-elafin or B. longum srp+ had lower CD3+ intraepithelial lymphocyte (IEL) counts in the small intestine than mice receiving vehicle and gliadin (P < 0.05) (Fig. 1B and C).

B. longum constitutively expressing srp exhibits an increased inhibitory capacity with respect to HNE activity in vitro. To characterize B. longum srp+, B. longum Δsrp, and B. longum srp+Con, we first measured the expression of srp in vitro. B. longum srp+Con expressed 452-fold more srp mRNA in vitro than B. longum srp+, and no srp mRNA was detected in B. longum Δsrp (Fig. 2A). We then quantified the ability of B. longum srp+ and B. longum srp+Con to inhibit human neutrophil elastase (HNE) activity in vitro, as pure SrP from B. longum srp+ was previously shown to inhibit HNE. B. longum Δsrp did not inhibit proteolysis of elastin by HNE, as levels of relative fluorescence units (RFU) produced from cleavage of fluorescein isothiocyanate-labeled elastin (FITC-elastin) were similar at all concentrations of added HNE. Compared to B. longum Δsrp, B. longum srp+ inhibited elastin degradation by HNE at 1.5 mU/ml (P < 0.01), resulting in a lower RFU value. B. longum srp+Con inhibited HNE activity at all concentrations of HNE compared to B. longum Δsrp (P < 0.01 at 3.125, 6.25, and 50 mU/ml; P < 0.05 at 1.5, 12.5, and 25 mU/ml) (Fig. 2B).

B. longum srp mediates the protective effect observed in mice. We tested the capacity of B. longum strains to prevent gliadin immunopathology using B. longum srp+, B. longum Δsrp (positive control), and B. longum srp+Con. NOD/DQ8 mice sen-
tized to gliadin and treated with \textit{B. longum} \textDelta srp had higher IEL counts than nonsensitized mice (controls; \( P < 0.0001 \)) or mice receiving \textit{B. longum} srp+ (\( P < 0.0001 \)) or \textit{B. longum} srp(Con) (\( P < 0.0001 \)) (Fig. 3A). Mice receiving \textit{B. longum} \textDelta srp had reduced villus-to-crypt (V:C) ratios compared with controls (\( P < 0.05 \)) and \textit{B. longum} srp(Con)-treated mice (\( P < 0.05 \)) (Fig. 3B). Lastly, mice treated with \textit{B. longum} \textDelta srp, but not mice receiving \textit{B. longum} srp+ or \textit{B. longum} srp(Con), had increased paracellular permeability in the proximal small intestine compared with controls (\( P < 0.05 \)) (Fig. 3C).

\textbf{Gliadin treatment and \textit{B. longum} srp expression shift fecal microbiota profiles in mice.} Both the small-intestinal and fecal contents of controls and gliadin-sensitized \textit{B. longum}-treated NOD/DQ8 mice were sequenced using 16S Illumina technology. The small-intestinal microbiota profiles were similar for all groups (see Fig. S1 and S2 in the supplemental material). However, in both weighted (Bray-Curtis dissimilarity) and
(Unifrac) β-diversity parameters, shifts in fecal microbiota profiles were observed between controls and all gliadin-sensitized mice (Fig. 4) (Fig. S3). Moreover, gliadin-sensitized mice treated with *B. longum* srp/H11001 and *B. longum* srp (Con) clustered separately from mice receiving *B. longum* srp/H9004, and this difference in β-diversity levels was significant (Fig. 4). Relative abundances of Actinomycetales were lower in all gliadin-sensitized mice than in controls. *B. longum* srp (Con) administration was associated with elevated levels of *Akkermansia*. The level of an unknown *Clostridiaceae* species was increased in mice treated with *B. longum* srp (Con) compared with those given *B. longum* srp− or no probiotic. The relative abundance of an unknown *Clostridiales* family XIII member was increased in gliadin-treated mice given *B. longum* Δsrp and *B. longum* srp (Fig. 5A and B).

*B. longum* and *B. longum* srp-expressing strains were detected in the gastrointestinal tract of treated mice. We next determined whether *B. longum* srp+ and the mutant strains, *B. longum* srp (Con) and *B. longum* Δsrp, were present in the small-intestinal lumen of treated mice via PCR amplification. There was no difference in the relative abundances of total *Bifidobacteria* in the small intestine between mice receiving vehicle or any of the *B. longum* strains, as measured by 16 illumina sequencing (Fig. 6A). However, strain-specific primers for *B. longum* srp+ revealed that *B. longum* srp+ and its derivatives were present in the small intestine of treated mice (Fig. 6B). Furthermore, srp mRNA was detected in the feces and/or intestinal content of 2/4 mice treated with srp+.
DISCUSSION

There is a spectrum of clinical conditions caused by adverse reactions to gluten and to its constitutive proteins, such as gliadin. These include the well-characterized autoimmune enteropathy CeD and wheat allergy as well as NCG/WS, which overlaps symptomatically with IBS (15). The only effective management for CeD is a lifelong...
gluten-free diet (GFD), which has several limitations, including poor compliance, accidental contaminations, and slow resolution of mucosal inflammation (16). Patients with NCG/WS also improve symptomatically on a GFD, but it is unknown whether these patients could tolerate less restrictive avoidance or could be successfully treated with other therapies. Since patients with active CeD and nonresponders to a GFD have been found to harbor dysbiotic intestinal communities (5, 17–19), probiotics have been proposed as potential candidates to restore gut microbial homeostasis. Smecuol et al. found that administration of the *Bifidobacterium* probiotic Natren Life Start (NLS) attenuated symptoms in CeD patients on a gluten-containing diet (20), and administration of NLS was shown to modulate innate immunity in a follow-up study (3). In another clinical trial, children with newly diagnosed CeD that received encapsulated *B. longum* CECT 7347 showed moderate changes in inflammatory markers and microbiota but no symptomatic improvement beyond those achieved with the concomitant GFD (21). Although these studies showed the possibility that certain probiotics, adjuvant to the GFD, may be beneficial in CeD and perhaps other gluten-related disorders, their use was not guided by a pathophysiological rationale and the mechanisms of action remain unclear.

Our study addressed the efficiency of a specific bacterial serpin (Srp), expressed naturally by *B. longum* srp*/H11001*, in the prevention of inflammation induced by gliadin in a genetically susceptible mouse model with previously determined well-defined endpoints (12, 22, 23). We have previously shown that the severity of gluten immunopathology in NOD/DQ8 mice is influenced by the microbiota with which these mice are colonized and that administration of recombinant *L. lactis* expressing elafin can attenuate the inflammatory response of the host to gluten (12, 23, 24). Serpins are produced by a wide range of organisms and play a key role in maintaining immune homeostasis (25, 26). In the gut, serpins are expressed at mucosal surfaces and are involved in regulating barrier function (27, 28). Srp inhibits eukaryotic serine proteases in vitro, including both neutrophil elastase and pancreatic elastase. The inhibition of neutrophil elastase, which is a driver of intestinal tissue damage and a biomarker of intestinal inflammation (29), represents an immunomodulatory capacity for Srp that may be relevant in treating gastrointestinal inflammatory conditions (13). We confirmed that the recombinant *B. longum* srp*(Con)* expresses higher levels of *srp* than *B. longum* srp* in vitro* (Fig. 2A) and that *srp* expression is undetectable in the mutant strain *B. longum* Δsrp in vitro. Since purified Srp from *B. longum* srp* has been demonstrated to inhibit...
HNE (13), we tested HNE inhibition by the three strains expressing srp at various levels in vitro. Indeed, B. longum strains expressing srp, but not B. longum Δsrp, inhibited HNE, suggesting that B. longum Srp has potential anti-inflammatory properties. Compared to B. longum Δsrp, B. longum srp(Con) inhibited HNE across all concentrations. Despite significant differences in srp expression between B. longum srp+ and B. longum srp- (Con), both strains inhibited HNE in our method of elastase activity quantification. More-significant inhibition of HNE by B. longum srp(Con) may be observed using alternative incubation times and alternative concentrations of elastase and substrate. Further, it was previously shown that elastase was capable of inducing serpin mRNA levels in wild-type B. longum strains (14). Such induction could explain why only limited differences between the two strains [B. longum srp+ and B. longum srp(Con)] were observed in this experiment. The innate immune response is a key component in the development of atrophy in CeD (30, 31) and has been proposed to be involved in the pathogenesis of NCG/WS (32, 33). The influx and release of neutrophil components are increased in patients with CeD (34), and by inhibiting HNE activity, Srp may specifically target a mechanism that contributes to gluten-related disorders.

Using the NOD/DQ8 model of gluten sensitivity, we examined the therapeutic potential of B. longum srp+ in vivo. As a quality control, we confirmed the presence of B. longum in the small intestine of probiotic-treated mice (Fig. 6B) and confirmed srp
expression only in mice receiving *B. longum* srp and *B. longum* srp(Con). Oral administration of *B. longum* srp and *B. longum* srp(Con) for 2 weeks protected mice from developing gliadin-induced immunopathology. Because these effects were not achieved in mice receiving *B. longum* Δsrp, srp expression is important for the protective mechanism. This may be related to immune regulation, maintenance of barrier function, overall beneficial shifts in gut microbiota, or inhibition of elastase released during inflammation (35, 36).

Probiotic-based therapies have been advocated to restore the balance of a “dysbiotic” or disease-promoting microbiota (6). Overgrowth of *Proteobacteria* in the small intestine has been reported in patients with active CeD and in those with persistent symptoms after gluten withdrawal (16, 23). We have shown that experimental expansion of levels of *Proteobacteria* in the small intestine of NOD/DQ8 worsens gluten immunopathology (16, 23). We therefore measured small-intestinal and fecal microbial β-diversity levels and relative abundances of bacterial groups (Fig. 4 and 5) (see Fig. S1 and S2 in the supplemental material). We found no significant shifts in the small-intestinal microbiota between the separate groups, suggesting that Srp from *B. longum* is unlikely to act through modification of compositional changes of the upper gastrointestinal tract microbiota (37–40). On the other hand, mild shifts in β-diversity were...
observed in fecal microbiota of mice treated with \textit{B. longum srp} and \textit{B. longum srp}(Con) compared to \textit{B. longum }\textit{srp}. Although most differences in relative abundances of genera between groups are difficult to interpret, the levels of \textit{Akkermansia} spp. were exclusively increased in \textit{B. longum srp}(Con)-treated mice compared to all other groups. The commensal \textit{Akkermansia muciniphila} is considered to be anti-inflammatory and beneficial for the intestinal mucus layer and barrier integrity in some models of inflammatory disorders (41, 42), and decreased levels of \textit{A. muciniphila} have been observed in patients with IBD and metabolic disorders (43, 44). This suggests the hypothesis that a significant level of Srp delivery, such as that provided by \textit{B. longum srp}(Con), may improve the overall performance of the mucosal barrier and immune function of the gut, in part through increases in the levels of \textit{Akkermansia} species. Because the role of \textit{Akkermansia} is somewhat controversial based on a recent study (45), the implications of this finding in our model must be further tested to draw conclusions.

In conclusion, \textit{B. longum srp} is a commensal bacterium that expresses a serpin, in a nonconstitutive manner, that is effective in preventing gliadin-induced immunopathology in NOD/DQ8 mice. As a commensal bacterium, \textit{B. longum srp} circumvents the controversy surrounding the use of GMOs for the delivery of anti-inflammatory molecules, which may facilitate its translation for human consumption. This report provides mechanistic insights and pathophysiological rationales to explore the efficacy of the use of \textit{B. longum srp} as an adjunctive therapy in gluten-related disorders or under other gastrointestinal inflammatory conditions associated with proteolytic imbalance. Future studies should address the host mechanisms behind the protection from gluten-induced pathology provided by protease inhibitors or behind exacerbation due to the presence of excess luminal proteases.

\section*{MATERIALS AND METHODS}

\textbf{Construction of bacterial strains}. \textit{B. longum} NCC2705 (\textit{B. longum srp} ) was isolated at the Nestlé Research Center from the feces of a healthy infant (46). The strain is well-characterized at the molecular and biochemical levels. The full genome of 2.26 Mb has been sequenced, and it was demonstrated that \textit{srp}, previously known as BI0108, encodes a bona fide serine protease inhibitor with affinity and inhibitory activity against eukaryotic elastases (13).

Upstream and downstream sequences (3 kb) of the \textit{srp} gene of \textit{B. longum srp} were amplified by PCR and cloned into the pJH101 vector. The pJH101 vector is available at the German Collection of Microorganisms and Cell Cultures (DSMZ) and was initially designed for the construction of integrable plasmids in \textit{Bacillus subtilis}. pJH101 contains a chloramphenicol resistance gene and does not contain an origin of replication for \textit{B. subtilis} or \textit{B. longum}. The resulting plasmid (pMDY24) containing no coding sequences of \textit{srp} was introduced into \textit{B. longum srp}. Transformation was performed as described previously (47). Five transformants were obtained by plating on De Man, Rogosa, and Sharpe (MRS) medium supplemented with 0.05\% cysteine (MRS-cys) containing chloramphenicol, and integration was confirmed by Southern blotting. Transformants were cultivated for 100 generations on MRS-cys without chloramphenicol to clear the antibiotic resistance gene. Twelve chloramphenicol-sensitive isolates were confirmed to be \textit{srp} knockout strains. One isolate (\textit{B. longum srp}) was included in the Nestlé Culture Collection under \textit{B. longum} NCC 9035.

Plasmid pMDY25 was constructed by inserting a constitutive promoter from \textit{B. longum} NCC 2705, pr-BL1363 (promoter of gene BI1363, coding for a glyceraldehyde 3-phosphate dehydrogenase), in front of the \textit{srp} gene in the pMDY23 plasmid which encodes spectinomycin resistance (48). In this recombinant strain (\textit{B. longum srp}(Con)), the level of synthesis of Srp no longer depends on any kind of induction.

The \textit{L. lactis} food-grade strain was engineered to express recombinant human elafin (\textit{L. lactis}-elafin), whose expression was driven by a nisin-inducible promoter, as described in detail previously (10). Strains and plasmids used in the manuscript are outlined in Table 1.

\textbf{Preparation of \textit{B. longum} biomass}. \textit{B. longum srp} and \textit{B. longum srp}(Con) strains were inoculated at 2\% from a fresh overnight culture in MRS-cys and grown anaerobically at 37°C for 16 h. Bacteria were harvested by centrifugation, resuspended in sterile phosphate-buffered saline (PBS) containing 20\% glycerol (PBS–20\% glycerol), and stored in aliquots at −80°C. Viable counts for the \textit{B. longum srp} and \textit{B. longum srp}(Con) preparations were equal to \(6.6 \times 10^6\) CFU/ml and \(4.4 \times 10^6\) CFU/ml, respectively. \textit{B. longum srp}(Con) was cultured and further processed as described above in the presence of 100 \(\mu\)g/ml of spectinomycin and grown for 48 h at 37°C. Levels of viable bacteria were equal to \(1.5 \times 10^9\) CFU/ml.

\textbf{In vitro inhibitory activity of \textit{B. longum} strains against elastase}. The enzymatic activity of human neutrophil elastase (HNE) was determined by cleavage of FITC-labeled elastin (FITC-elastin). Concentrations of HNE (1.5, 3.125, 6.25, 12.5, 25, and 50 \(\mu\)l/m) were incubated with 10\% CFU of \textit{B. longum srp}, \textit{B. longum srp}(Con), or \textit{B. longum srp}(Con) and 40 \(\mu\)l of buffer solution (50 mM Tris-HCl, 1 mM CaCl\(_2\), 50 mM NaCl, 0.25\% Triton X-100; pH 8.0) at 37°C for 30 min. FITC-elastin substrate (50 \(\mu\)l) was added, and

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TABLE 1 Bacterial strains used and plasmids employed to construct them

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description (reference or source)</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>L. lactis</em>-elafin</td>
<td>Recombinant <em>Lactococcus lactis</em> strain expressing elafin (1)</td>
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<tr>
<td><em>B. longum</em> srp²</td>
<td>Commensal probiotic strain <em>Bifidobacterium longum</em> NCC 2705 expressing serpin (2)</td>
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<tr>
<td><em>B. longum</em> srp(Con)</td>
<td>Recombinant <em>Bifidobacterium longum</em> strain derived from NCC 2705 (NCC 2705 pMDY25), constitutively producing serpin (this work)</td>
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<tr>
<td><em>B. longum</em> Δsrp</td>
<td>Genetically modified <em>Bifidobacterium longum</em> strain derived from NCC 2705 (NCC 9035) without serpin coding sequences (this work)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pJH101</td>
<td>Commercially available plasmid (DSMZ) initially designed for the construction of integrable plasmids in <em>Bacillus subtilis</em>; contains a chloramphenicol resistance gene and does not contain origin of replication for either <em>Bacillus subtilis</em> or <em>Bifidobacterium longum</em></td>
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<tr>
<td>pMDY24</td>
<td>Plasmid derived from pJH101 containing upstream and downstream sequences of the BL108 serpin gene and containing chloramphenicol resistance gene (this work)</td>
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<tr>
<td>pMDY23</td>
<td>Versatile reporter plasmid based on <em>Bifidobacterium longum</em> cryptic plasmid and <em>Escherichia coli</em> gusA gene and containing spectinomycin resistance gene (3)</td>
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<tr>
<td>pMDY25</td>
<td>Plasmid derived from pMDY23 containing a constitutive promoter in front of the BL108 serpin gene (this work)</td>
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fluorescence was measured at an excitation wavelength of 530 nm using a spectrophotometer (SpectraMax; Molecular Devices, San Leandro, CA).

**Animals.** All experiments were conducted with approval from the McMaster University Animal Care Committee. Female and male 8-to-12-week-old NOD/DQ8 transgenic mice (22) were fed a gluten-free diet for 2 generations (Harlan Laboratories, Indianapolis, IN) and housed in a specific-pathogen-free colony at McMaster University. These mice lack all endogenous mouse major histocompatibility complex class II (MHC II) molecules and express the DQ8 human transgene on a NOD background (22). Oral sensitization of NOD/DQ8 mice with peptic-tryptic (PT) digestion of gliadin, one of the main protein fractions in gluten, and subsequent gliadin challenge induce moderate enteropathy, intraepithelial lymphocytosis, and barrier dysfunction (Fig. 1A) as described previously (22).

**Mucosal delivery of *B. longum* and gliadin sensitization.** Mice were subjected to oral gavage with 500 µg PT-gliadin plus 25 µg choler toxin as an adjuvant (Sigma-Aldrich) once a week for 3 weeks. Nonsensitized mice (controls) were subjected to gavage with PBS plus 25 µg choler toxin. Sensitized mice were then treated daily by oral gavage (10⁹ CFU, 200 µl/mouse) for 2 weeks with *B. longum* srp² or *B. longum* Δsrp or *B. longum* srp(Con) suspended in PBS–20% glycerol. During the probiotic treatment period, sensitized mice were orally challenged with gliadin (2 mg/mouse) dissolved in 0.02 M acetic acid (vehicle) three times per week. Vehicle-treated mice were simultaneously subjected to gavage with PBS–20% glycerol during the challenge period. Control mice were maintained on a gluten-free chow diet and subjected to gavage with PBS–20% glycerol and 0.02 M acetic acid (Fig. 1A).

**Detection of *B. longum* strains.** Primers associated with specificity for *B. longum* srp² and derivatives, targeting the previously described insertion sequence IS8106 (49), were used to amplify DNA extracted from proximal small-intestinal tissue and contents (Fig. 6B). The primers were as follows: forward, 5'–TCCAGATCATTTCCGATTC–3'; reverse, 5'–CGCGCATTTTCTATGCTC–3'. The primers were amplified as previously described (49).

**srp mRNA expression in *B. longum* strains.** *B. longum* srp², *B. longum* srp(Con), and *B. longum* Δsrp were cultured as described above for 8 h, and cells were collected by centrifugation. Total RNA was extracted using an RNeasy minikit (Qiagen) with additional DNase treatment. Purity and quality were checked using QIAxcel RNA quality control kit v2.0 (Qiagen). RNA level was quantified using a SuperScript III Platinum SYBR green One-Step quantitative real-time PCR (qRT-PCR) kit (Invitrogen) and the standard PCR conditions described in the kit instructions. The *srp* primers were as follows: forward, 5’–ACAAATCGCTGTAATGTTCCG–3'; reverse, 5’–TCGGTGGCAAGAGTAGTGC–3'. The lactate dehydrogenase (*ldh*) house-keeping gene was used for standardization. The following primers were used for *ldh*: forward, 5’–CGAAGCCCATCTACATGCTC–3'; reverse, 5’–AAAGATCGTGGTTCTTGGAC–3'. Fold changes of *srp* mRNA levels were calculated using the Pfaffl method (50).

**srp detection in vivo.** *srp* mRNA levels were measured in small-intestinal contents and feces of *B. longum* srp²-treated mice. Samples were collected fresh and flash frozen in liquid nitrogen. RNA was extracted from these samples using a PowerMicrobiome RNA isolation kit (catalog no. 26000-50; MoBio Laboratories Inc., Carlsbad, CA). RNA quality was checked using an Agilent 2100 Bioanalyzer system with an Agilent RNA 6000 Nano kit. RNA was quantified using a Quant-it Ribogreen RNA kit. *srp* mRNA levels were calculated using an Agilent 2100 Bioanalyzer system with an Agilent RNA 6000 Nano kit. RNA was quantified using a Quant-it Ribogreen RNA kit. *srp* mRNA levels were calculated using the Pfaffl method (50).
were measured by qRT-PCR in two steps. RNA transcription to single-stranded cDNA was performed using 1 μg of RNA in a total reaction mixture of 20 μl by the use of Ambion qScript cDNA supermix and the following PCR conditions: 25°C for 5 min; 42°C for 30 min; 85°C for 5 min; a 4°C hold. spn was further amplified by real-time PCR using the following primers and probe: forward primer, 5’-ACCAATCGCTGC TAA GTCG-3’; reverse primer, 5’-TCCGTGCAAGAGATGAC-3’; probe, 5’-6-carboxyfluorescein (FAM)- CCGAGATGAGCGCCCGAAGT-black hole quencher (BHQ [Microsynth])-3’. cDNA (100 ng) was used in a total reaction mixture of 20 μl with the TaqMan Universal PCR master mix and the following cycle: 50°C for 2 min; 95°C for 10 min; 95°C for 15 s; 60°C for 1 min (repeated for 40 cycles).

Evaluation of small-intestinal immunopathology. Smallintestinal cross sections were fixed in 10% buffered formalin for 48 h and embedded in paraffin, as previously described (22). Immunohistochemistry experiments were performed on formalin-fixed, paraffin-embedded sections of proximal small intestine to visualize CD3+ cells as described previously (22, 51). Slides were examined at ×20 magnification using light microscopy in a blind fashion. The number of CD3+ IELs per 20 enterocytes in five randomly chosen villous tips was counted by an observer in a blind fashion as described above, and data were expressed as numbers of IELS/100 enterocytes (51). Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for histological evaluation of tissue morphology under conditions of light microscopy (Olympus, Ontario, Canada). Using Image-Pro 6.3 software (Medicybernetics, MD, USA), enteropathy was quantified in a blind fashion by measuring villus-to-crypt (V:C) ratios as previously described (22). Intestinal paracellular permeability was evaluated ex vivo by the use of theUsing chamber technique as previously described (World Precision Instruments, Sarasota, FL) (22). Paracellular permeability of proximal small intestinal samples was evaluated by measuring the mucoosa-to-serous flux of the inert paracellular probe 51Cr-EDTA. 51Cr-EDTA was quantified in samples using a liquid scintillation counter and expressed as percent recovery of 51Cr-EDTA flux per square centimeter per hour.

Microbiota compositional analysis. Fecal and small-intestinal contents were collected and flash frozen on dry ice. DNA was extracted from samples as previously described (52) and amplified for the hypervariable V3 region of the 16S rRNA gene for sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA). Analysis of data was performed as previously described (52). Briefly, sequences were trimmed using Cutadapt software (version 1.2.1) (53) and aligned using PANDAseq software (version 2.8) (54). Operational taxonomic units selected using AbundantOTU (55) were assigned taxonomy according to the Greengenes reference database (56). Principal-coordinate analysis (PCoA) plots were generated using R (R Foundation for Statistical Computing, Vienna, Austria). Pairwise UniFrac distances were calculated among microbial communities, and both relative abundance data (weighted) and presence/absence information (unweighted) were determined.

Statistical analysis. Data were analyzed using GraphPad Prism 6.0, QIIME, R and SPSS software. Normal data were analyzed by analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. Statistical analyses of HNE inhibition compared to buffer results were performed using the Mann-Whitney test. Statistical analyses of microbiota β-diversity were performed using permutational multivariate analysis of variance (PERMANOVA). Microbiota abundances were analyzed in SPSS via Kruskal-Wallis testing followed by false-discovery-rate (FDR) determinations (q < 0.05). All significant genera presented passed FDR testing.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01323-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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