

Peptidoglycan-Chi3l1 interaction shapes gut microbiota in intestinal mucus layer

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Abstract

The balanced gut microbiota in intestinal mucus layer plays an instrumental role in the health of the host. However, the mechanisms by which the host regulates microbial communities in the mucus layer remain largely unknown. Here, we discovered that the host regulates bacterial colonization in the gut mucus layer by producing a protein called Chitinase 3-like protein 1 (Chi3l1). Intestinal epithelial cells are stimulated by the gut microbiota to express Chi3l1. Once expressed, Chi3l1 is secreted into the mucus layer where it interacts with the gut microbiota, specifically through a component of bacterial cell walls called peptidoglycan. This interaction between Chi3l1 and bacteria is beneficial for the colonization of bacteria in the mucus, particularly for gram-positive bacteria like *Lactobacillus*. Moreover, a deficiency of Chi3l1 leads to an imbalance in the gut microbiota, which exacerbates colitis induced by dextran sodium sulfate (DSS). By performing fecal microbiota transplantation from Villin-cre mice or replenishing *Lactobacillus* in IEC^{ΔChi3l1} mice, we were able to restore their colitis to the same level as that of Villin-cre mice. In summary, this study shows a “scaffold model” for microbiota homeostasis by interaction between intestinal Chi3l1 and bacteria cell wall interaction, and it also highlights that an unbalanced gut microbiota in the intestinal mucus contributes to the development of colitis.

eLife assessment

Supported by **convincing** data, this **valuable** study demonstrates that the Chitinase 3-like protein 1 (Chi3l1) interacts with gut microbiota and protects animals from intestinal injury in laboratory colitis model. The revised manuscript sufficiently addressed the reviewers' comments. The work will be of interest to scientists studying crosstalk between gut microbiota and inflammatory diseases.

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Introduction

Intestinal homeostasis is crucial for maintaining human health[1]. Alterations in gut microbiota composition have been linked to various diseases including cancer, obesity, and neurological disorders[2–6]. Dysbiosis, which refers to an imbalance in gut microbiota, is characterized by decreased microbial diversity, the presence of harmful microbes, or absence of beneficial ones [7]. Therefore, understanding the factors that influence gut microbiota is a fundamental goal in microbiome research[8]. Growing evidence suggests that colonization of the gut mucus layer can affect the susceptibility and progression of intestinal diseases like inflammatory bowel disease (IBD), irritable bowel syndrome, and celiac disease[9]. Inflammatory bowel diseases, such as Crohn's disease (CD) and Ulcerative Colitis (UC), are characterized by chronic inflammation of the intestinal mucosa. Although the cause of the inflammatory bowel disease is unclear, mouse models lacking the key components of the mucus are predisposed to colitis, accompanied by dysbiosis in mucosa[10, 11], which is in accordance with increased epithelial-adherent microbial communities in biopsies from patients with IBD [12, 13]. Furthermore, there were significant differences in the gut microbiota of CD patients compared with healthy controls, and these differences were only present in mucus samples (not stool samples), suggesting that bacteria in the mucus layer may be more important for the development of IBD[14]. Donaldson et al. discovered that the intestinal flora utilizes host immunoglobulin A (IgA) for mucus colonization, indicating that the host may secrete certain factors to maintain intestinal flora homeostasis in the mucus[15]. However, the mechanisms regulating gut microbiota colonization in the mucus layer remain largely unknown. We aim to investigate the regulation of microbial communities in gut mucus and its implications in intestinal diseases.

Chitinase 3-like protein 1 (Chi3l1, also known as YKL-40 in humans) is a secreted protein that belongs to the glycosylhydrolase 18 family[16]. Despite its name, Chi3l1 can bind to chitin but does not have chitinase activity[17]. In our investigation, we noticed that chitin and peptidoglycan, a major component of bacterial cell walls, have similar structures [18]. Based on this information, we speculate that Chi3l1 might also interact with peptidoglycan and, therefore, interact with bacteria. Interestingly, Chi3l1 is expressed in intestinal epithelial cells and the lamina propria. We hypothesize that Chi3l1 may be secreted by intestinal epithelial cells and regulate the gut microbiota through its interaction with peptidoglycan in the mucous layer. In our study, we discovered that gut microbiota induce the expression of Chi3l1 in epithelial cells. Once expressed, Chi3l1 is secreted into the mucosa and interacts with bacteria, particularly with the bacterial cell wall component peptidoglycan. This interaction promotes bacterial colonization, especially of beneficial bacteria such as *Lactobacillus*. As a result, mice with higher levels of Chi3l1 are less susceptible to colitis.

Results

Intestinal epithelial cells express Chi3l1 induced by gut microbiota

The gut microbiota's composition is shaped by host factors, including IgA [15], RegIIIγ [19], and TLR-5 [18]. Yet, specific host factors which maintain the homeostasis of the microbiota remain largely undefined. Drawing on the theory of co-evolution between the host and microbiota [20], we propose that host factors, which are induced by bacteria in the gut, could play a pivotal role in regulating bacterial colonization and composition. In a previous study, it was observed that both live *E.coli* and heat-killed *E.coli* treatment resulted in a significant increase in the expression of the gene encoding Chi3l1 in human intestinal organoids[21]. To verify this finding, we conducted immunohistochemical staining on intestinal tissue sections of germ-free and specific

pathogen free (SPF) C57BL/6J wildtype mice. We observed a substantial increase of Chi3l1 expression in SPF mice compared to germ-free mice (**Fig. 1A**). The intestinal epithelium comprises various cell types, including intestinal cells, goblet cells, enteroendocrine cells, Tuft cells, Paneth cells, M cells, and others [21]. To identify the cellular sources of Chi3l1, we performed co-staining with markers for specific cell types, including Chromogranin A (ChgA) for enteroendocrine cells, Ulex Europaeus Agglutinin I (UEA-1) for goblet cells and Paneth cells, and Double cortin-like kinase 1 (DCLK1) for tuft cells. Our results revealed that Chi3l1 is primarily expressed in enteroendocrine cells in the ileum and goblet or Paneth cells in the colon (**Fig. 1B**). However, Chi3l1 expression was not observed in tuft cells (Supplementary Figure 1).

Furthermore, we isolated total bacteria from wildtype mouse feces and treated DLD-1 cells (a colorectal adenocarcinoma cell line) with the bacterial extract for 12 hours. We found that the bacterial extract directly induced Chi3l1 expression in DLD-1 cells (**Fig. 1C**). To examine whether the induction of Chi3l1 expression require a specific bacteria strain, we further identified the bacterial extract using 16S rRNA sequencing. Our results revealed that *E.coli* specifically stimulated Chi3l1 expression in DLD-1 cells, while *Staphylococcus Sciuri* did not have the same effect (**Fig. 1D**). Although our data are limited to these two bacterial strains, it suggest that not all bacteria can induce the expression of Chi3l1. Next, we wondered what component of bacteria can induce Chi3l1 expression. We tried heat-killed *E.coli*, which maintains bacterial cell wall integrity. We found that treatment of DLD-1 cells with heat-killed *E.coli* also led to an induction of Chi3l1 expression (**Fig. 1E**). These findings suggest that the induction of Chi3l1 expression does not necessarily require live bacteria and that bacterial components alone are sufficient to induce this response.

Given that *E. coli* is Gram-negative and *Staphylococcus sciuri* is Gram-positive, we hypothesized that the difference in their ability to induce Chi3l1 expression might be due to variations between Gram-negative and Gram-positive bacteria, such as the presence of lipopolysaccharides (LPS). To test this hypothesis, we used LPS to induce Chi3l1 expression. Consistent with our hypothesis, LPS successfully induced Chi3l1 expression (**Fig. 1F** & **G**). Collectively, these findings provide evidence that the gut microbiota can induce Chi3l1 expression in intestinal epithelial cells. Collectively, these findings provide evidence that the gut microbiota can induce Chi3l1 expression in intestinal epithelial cells.

Chi3l1 interact with bacteria via peptidoglycan

Chi3l1 belongs to a group of proteins called non-enzymatic chitinase-like proteins, which are known to bind chitin. Chitin is a polysaccharide present in the exoskeleton of arthropods and the cell walls of fungi [22]. By comparing the structure of chitin with that of peptidoglycan (PGN), a component of bacterial cell walls, we observed that they have similar structures (**Fig. 2A**). Based on this observation, we hypothesized that Chi3l1 may interact with gut bacteria through PGN. To test our hypothesis, we conducted co-incubation experiments where we mixed recombinant mouse Chi3l1 (rmChi3l1) with either Gram-positive or Gram-negative bacteria and then precipitated the bacteria through centrifugation. We found that rmChi3l1 was present in the pellet obtained from both Gram-positive and Gram-negative bacteria (**Fig. 2B**), suggesting that Chi3l1 can directly interact with bacteria.

To further investigate the interaction between Chi3l1 and PGN, we also co-incubated PGN with rmChi3l1 and precipitated the PGN through centrifugation. PGN is an insoluble substance and hence can be precipitated by centrifugation. Consistent with our previous results, we observed that rmChi3l1 was present in the pellet obtained from PGN but not in the pellet obtained from bovine serum albumin (BSA), which served as a negative control (**Fig. 2C**). Furthermore, we also examined the interaction between PGN and recombinant human Chi3l1 (rhChi3l1) and obtained similar results (**Fig. 2D**). These findings indicate that Chi3l1 interacts with bacteria through PGN.

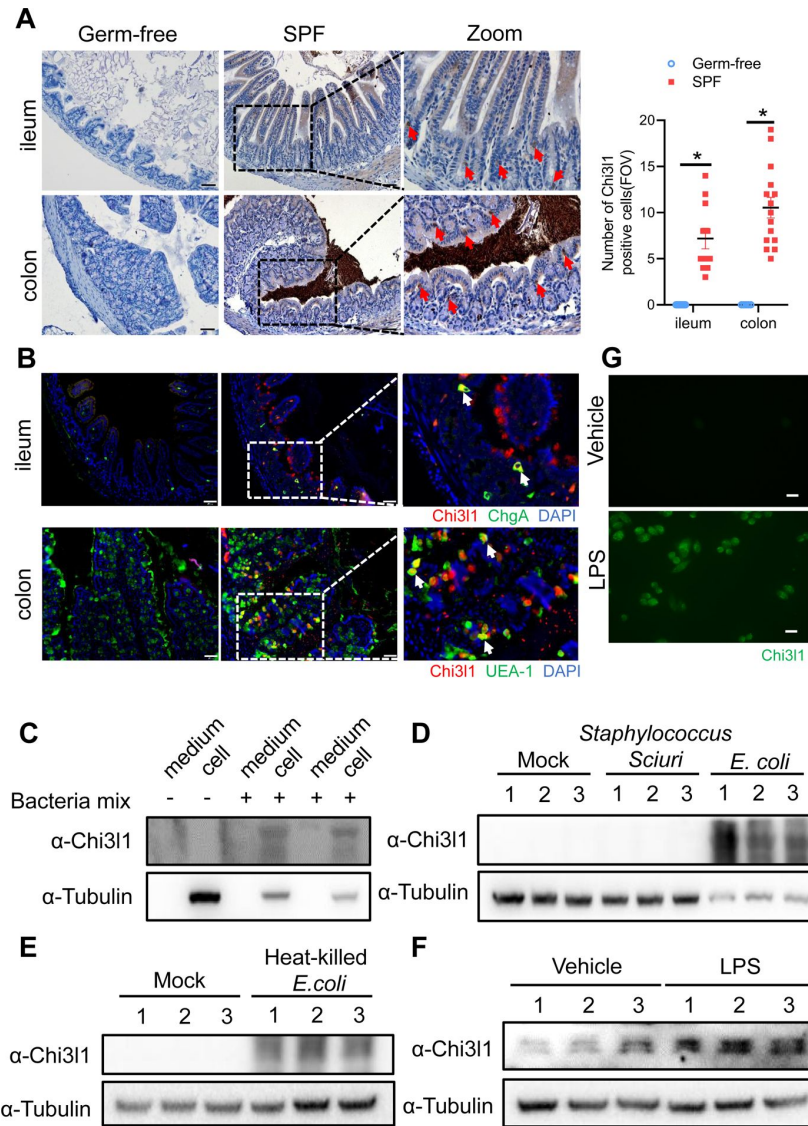


Figure 1.

Intestinal epithelial cells express Chi3l1 induced by gut microbiota.

(A) IHC staining to detect Chi3l1 in both ileum and colon from germ-free and wildtype mice. Ctrl (wildtype mice without application of first antibody), WT (wildtype C57B/6j mice). Red arrows indicate Chi3l1-expressing cells. Scale bar, 50 μ m (Ctrl, Germ-free, WT) and 20 μ m(zoom). The number of Chi3l1-positive cells in each field of view (FOV) was analyzed. **(B)** Ileum and colon were collected from wildtype mice and stained with ChgA (green), Chi3l1(red), and nuclear DAPI (blue) in ileum and UEA-1 (green), Chi3l1(red), and nuclear DAPI (blue) in colon. Scale bar, 20 μ m. Ctrl (without application of first antibody), WT (wildtype C57B/6j mice). **(C)** Western blot to detect Chi3l1 protein expression in DLD-1 cells after bacteria mix infection for 12 hour. Bacteria mix are total bacteria extracted from feces of wildtype mice. **(D)** Western blot to detect Chi3l1 protein expression in DLD-1 cells after *Staphylococcus Sciuri* and *E. coli* infection for 12 hour. *Staphylococcus Sciuri* and *E. coli* are isolated from bacteria mix and verified by 16S rRNA sequencing. Three independent experimental results are showed. **(E)** Western blot to detect Chi3l1 protein expression in DLD-1 cells after treatment with heat-killed *E. coli* for 12 hour. Three independent experimental results are showed. **(F)** Western blot to detect Chi3l1 protein expression in DLD-1 cells after treatment with 100 pg/ml LPS for 12 hour. Three independent experimental results are showed. **(G)** Immunofluorescence to detect Chi3l1 protein expression in DLD-1 cells after treatment with 100pg/ml LPS for 12 hour. Scale bar, 20 μ m. The presence of cells in the untreated sample are annotated using white dashed lines based on the overexposure. All data above represent at least three independent experiments. Representative images are shown in A,B, n=3-4 mice/group.

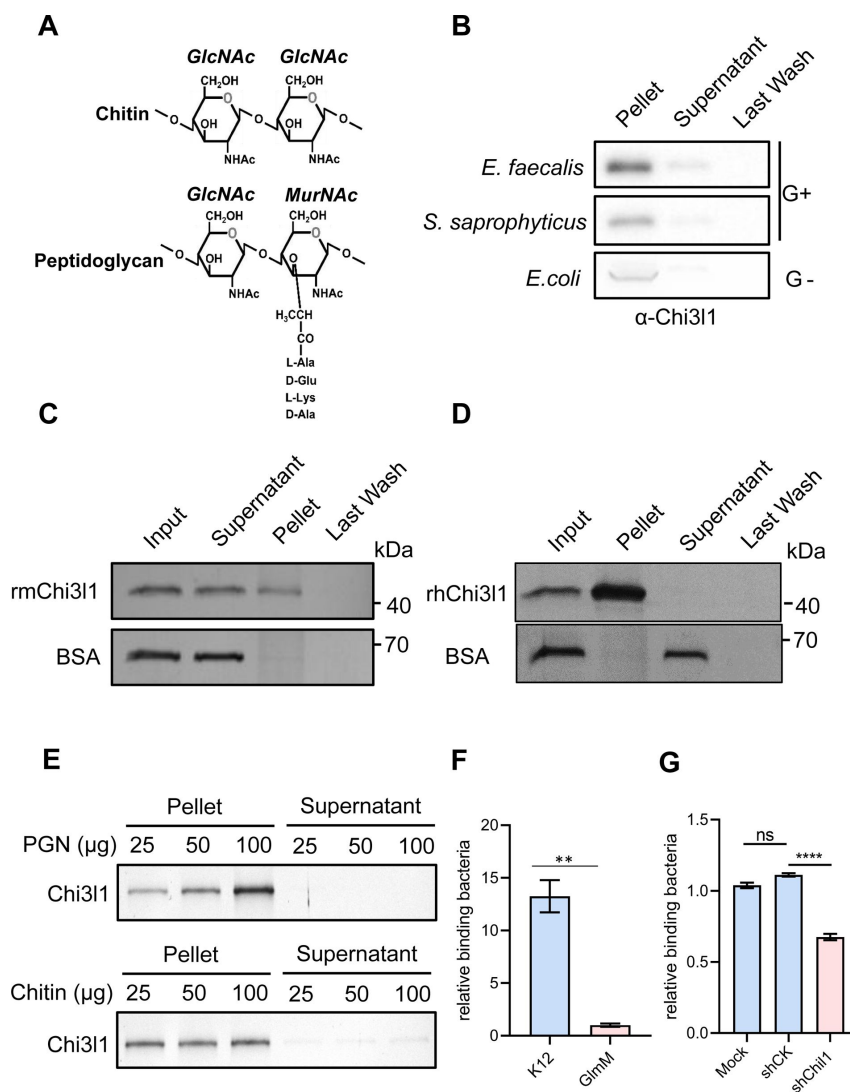


Figure 2.

Chi3l1 interact with bacteria via peptidoglycan.

(A) Structural comparison between Chitin and peptidoglycan. Both chitin and peptidoglycan contain N-acetylglucosamine (GlcNAc) and have β -1,4-glycosidic bonds in their structures. However, Chitin is purely a polysaccharide, while peptidoglycan includes a peptide component that forms cross-links between chains. [30] **(B)** Gram-positive bacteria (*E. faecalis*, *S. saprophyticus*) and Gram-negative bacteria (*E. coli*) were incubated with 1 μ g of recombinant mouse Chi3l1 protein (rmChi3l1), respectively. Proteins bound to indicated bacteria were precipitated by centrifugation. Western blot was used to detect rmChi3l1 in Pellet, Supernatant (unbound proteins) and Last Wash (last wash unbound proteins). **(C)** Insoluble Peptidoglycan (PGN) were incubated with either recombinant mouse Chi3l1 protein (rmChi3l1) or Bovine serum albumin (BSA). Proteins bound to PGN were precipitated by centrifugation. Silver staining was used to detect rmChi3l1 in Input, Supernatant (unbound proteins), Pellet and Last Wash (last wash unbound proteins). **(D)** Insoluble Peptidoglycan (PGN) were incubated with recombinant human Chi3l1 protein (rhChi3l1). Proteins bound to PGN were precipitated by centrifugation. Silver staining was used to detect rhChi3l1 in Input, Supernatant (unbound proteins), Pellet and Last Wash (last wash unbound proteins). All data above represent at least three independent experiments. **(E)** Insoluble PGN or chitin was incubated with rmChi3l1. Chi3l1 bound to PGN (upper panel) and chitin (lower panel) was precipitated and detected by silver staining. The supernatant represents the last wash, and the pellet contains proteins precipitated by either PGN or chitin. **(F)** Relative DLD-1 bacterial binding preference after treatment with K12 or GlnM, a PGN synthesis-deficient mutant. Colony-forming units (CFU) were counted, and GlnM CFU were normalized to 1. **(G)** Relative K12 bacterial adhesion preference after DLD-1 cells were transfected without (Mock), or with scramble shRNA (shCK), or with shChi1. CFU were counted, and the Mock group were normalized to 1.

To better characterize the binding between Chi3l1 and peptidoglycan (PGN), we compared the binding affinities of Chi3l1 to both PGN and chitin. We incubated chitin and PGN with rmChi3l1 in increasing doses (25, 50, 100 μ g) and detected the precipitated rmChi3l1 by either chitin or PGN. Our results indicate that Chi3l1 interacts with PGN in a dose-dependent manner (**Figure 2E**). In contrast, the binding between Chi3l1 and chitin did not exhibit dose dependency (**Figure 2E**). These findings suggest a specific and distinct binding mechanism for Chi3l1 with PGN compared to chitin.

To investigate whether the Chi3l1-PGN interaction could facilitate the colonization of gram-positive bacteria, we conducted adhesion experiments using DLD-1 cells and bacteria. We employed a GlmM mutant (PGN synthesis-deficient) and K12 bacteria (a wild-type *E. coli* strain used as a control) to assess their adhesion capabilities. The results showed that the adhesion ability of the GlmM mutant to cells significantly decreased (**Figure 2F**). Additionally, after knocking down Chi3l1 in DLD-1 cells (knockdown efficiency over 50%), we observed a decrease in bacterial adhesion (**Figure 2G**). These findings suggest that the Chi3l1-PGN interaction plays a crucial role in bacterial adhesion.

Intestinal bacteria are disordered in IEC ^{Δ Chi1} mice, especially Gram-positive bacteria

To gain initial insights into how the expression of Chi3l1 in intestinal epithelial cells (IECs) affects the colonization of gut microbiota, we created mice with a specific deficiency of Chi3l1 in IECs (referred to as IEC ^{Δ Chi1} mice) (Supplementary Figure 2). We then conducted bacterial 16S rRNA sequencing analysis of the colon contents of both Villin-cre and IEC ^{Δ Chi1} littermates. Our analysis of alpha diversity revealed that the bacterial population was relatively lower in IEC ^{Δ Chi1} littermates compared to Villin-cre littermates (**Figure 3A**). This finding was further confirmed by conducting universal bacterial 16S rRNA qPCR analysis of the feces and ileum contents of IEC ^{Δ Chi1} and Villin-cre littermates, which also showed lower bacterial enrichment in IEC ^{Δ Chi1} mice (**Figure 3B**). Furthermore, principal component analysis demonstrated significant differences in bacterial diversity between Villin-cre and IEC ^{Δ Chi1} littermates (**Figure 3C**).

When we examined the relative abundance of Gram-positive and Gram-negative bacteria between Villin-cre and IEC ^{Δ Chi1} littermates, we observed that Gram-positive bacteria were significantly reduced in IEC ^{Δ Chi1} mice, while there was no notable difference in Gram-negative bacteria (**Figure 3D**). This result was further validated by staining lipoteichoic acid (LTA), a component present on Gram-positive bacteria, which revealed a lower abundance of Gram-positive bacteria in IEC ^{Δ Chi1} compared to Villin-cre littermates (**Figure 3E**). Moreover, visualization of *Firmicutes* by bacteria FISH staining, a dominant group of Gram-positive bacteria in the gut, also showed reduced levels of *Firmicutes* in the colon lumen of IEC ^{Δ Chi1} mice compared to Villin-cre mice (**Figure 3F**). Analysis of the relative abundance of specific Gram-positive bacterial species demonstrated a significant reduction in *Lactobacillus* in IEC ^{Δ Chi1} mice compared to Villin-cre mice (**Figure 3G**). Similar results were observed in Chi1^{-/-} mice compared to wildtype mice (**Figure 3H**). Consistent with the 16S rRNA sequencing analysis data, qPCR results showed that *Turicibacter* was more abundant in IEC ^{Δ Chi1} mice than Villin-cre mice (Supplementary Figure 3A). These findings suggest that Chi3l1 plays a role in regulating the colonization of Gram-positive bacteria, particularly *Lactobacillus*, in the murine gut.

Chi3l1 promotes the colonization of Gram-positive bacteria in intestinal mucus

Chi3l1 was found in secretory cells like goblet cells and Paneth cells, suggesting that it may be secreted into the intestinal lumen (**Fig. 1B**). Immunohistochemistry staining of Chi3l1 in the colon revealed a large amount of Chi3l1 signals in the mucus layer (**Fig. 4A**). Immunofluorescence co-staining of Chi3l1 with UEA-1 in the colon yielded similar results (**Fig.**

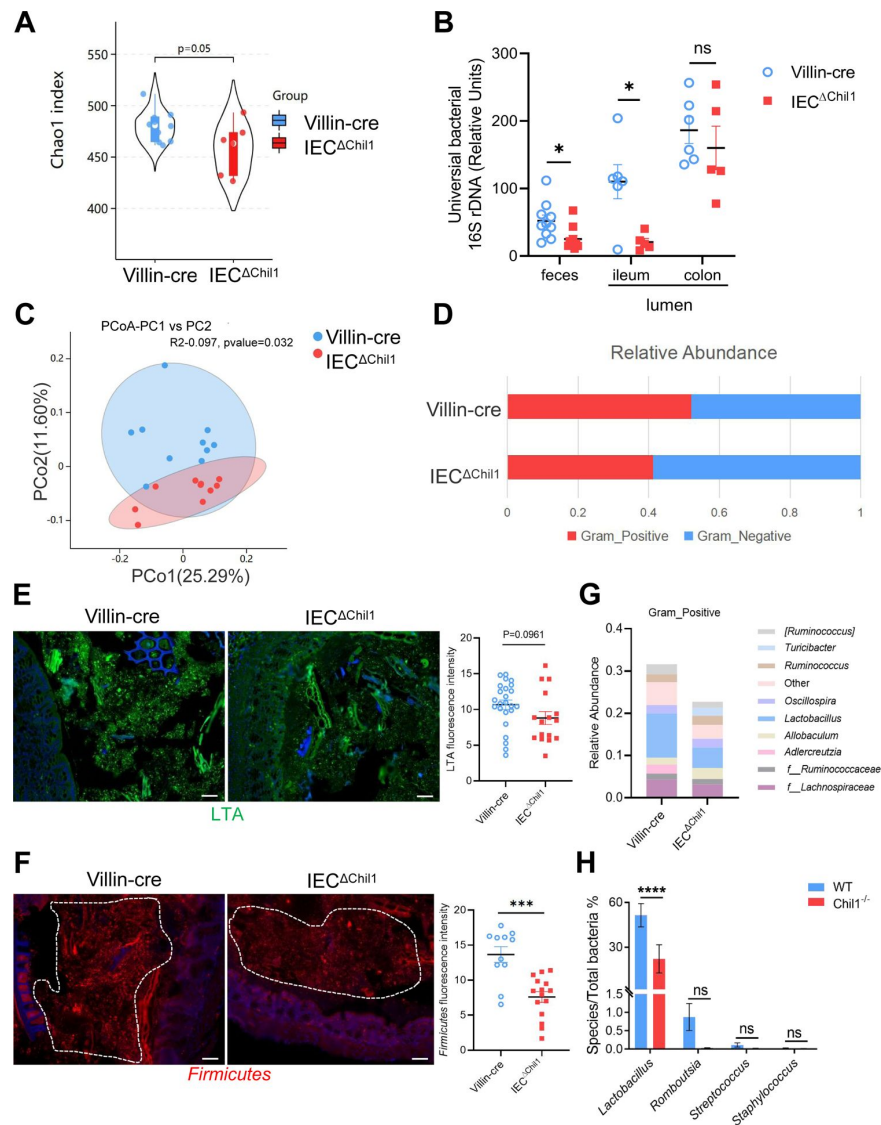


Figure 3.

Intestinal bacteria are disordered in IEC Δ Chil1 mice, especially Gram-positive bacteria.

(A, C, D, G) Female Villin-cre and IEC Δ Chil1 littermates continue to cage together after weaning for 8 weeks. Microbial communities in feces and intestinal lumen were characterized by 16S rRNA sequencing. $n=7$ or 10 /group. (A) Alpha diversity analysis of colon contents between Villin-cre and IEC Δ Chil1 littermates. (B) qPCR analysis of total bacteria in the feces and ileum, colon luminal microbial communities of Villin-cre and IEC Δ Chil1 littermates. Values for each bacterial group are expressed relative to total 16S rRNA levels. Mean $\pm$ SEM is displayed. Two-tailed, unpaired student t-test was performed. * $P < 0.05$, ns, not significant. $n=5-10$ /group. (C) Principal Component Analysis of weighed UniFrac distances of 16S community profiles of Villin-cre and IEC Δ Chil1 littermates feces (binary-jaccard). (D) Relative abundance of Gram-positive and Gram-negative bacteria in colon contents of Villin-cre and IEC Δ Chil1 littermates are shown. (E) LTA (green) was detected by immunofluorescence in colon sections of Villin-cre and IEC Δ Chil1 littermates. Nuclei were detected with DAPI. Scale bar, $50\mu\text{m}$. The average fluorescence intensity in each field of view (FOV) was analyzed. (F) Fluorescence in situ hybridization (FISH) detection of gram-positive bacteria (red) in the colon of Villin-cre and IEC Δ Chil1 littermates, nuclei were detected with DAPI (blue). Scale bars, $50\mu\text{m}$. The average fluorescence intensity in each field of view (FOV) was analyzed. (G) Relative abundance of Gram-positive bacteria genera in colon lumen of Villin-cre and IEC Δ Chil1 littermates. (H) Female wildtype and Chil1 $^{-/-}$ littermates continue to cage together after weaning for 8 weeks. Microbial communities in feces were characterized by 16S rRNA sequencing. Mean $\pm$ SEM is displayed. Two-tailed, unpaired student t-test was performed. **** $P < 0.0001$, ns, not significant. $n=4$ or 6 /group. Representative images are shown in E, F, $n=3/4$ mice/group.

4B). Furthermore, Chi3l1 was also detected in the ileum and colonic tissues and mucus layer (Fig. 4C & D). These findings indicate that mouse Chi3l1 is specifically expressed in intestinal secretory epithelial cells and secreted into the intestinal lumen. Since large amounts of Chi3l1 is secreted into the mucus and Chi3l1 interact with bacteria, we hypothesize that Chi3l1 may regulate the colonization of Gram-positive bacteria in the mucus layer. To test this hypothesis, we isolated bacterial DNA from the ileum and colon mucus of both wildtype and Chi11^{-/-} mice. Quantification of Gram-positive bacteria and *Lactobacillus* using qPCR revealed that both the ileum and colon mucus of Chi11^{-/-} mice had significantly lower levels of Gram-positive bacteria and *Lactobacillus* compared to that of wildtype mice (Fig. 4E & F).

To further validate these results, we labeled a Gram-positive bacteria strain, *E. faecalis*, with fluorescent D-amino acids (FDAA), which can metabolically label bacterial peptidoglycans[23]. We then performed rectal injection of both wildtype and Chi11^{-/-} mice with FDAA-labeled *E. faecalis*. The data demonstrated that Chi11^{-/-} mice had much lower colonization of *E. faecalis* compared to wildtype mice (Fig. 4G). Besides Gram-positive bacteria, we also performed rectal injection of both wildtype and Chi11^{-/-} mice with mCherry-OP50 (a strain of *E. coli* that express mCherry), we found Chi11^{-/-} mice had much higher colonization of *E. coli* compared to wildtype mice (Supplementary Figure 3B). Based on these findings, we conclude that Chi3l1 promotes the colonization of Gram-positive bacteria, particularly *Lactobacillus*, in the intestinal mucus. Additionally, we also observed that the deletion of Chi3l1 significantly reduced mucus layer thickness, which may be attributed to the disrupted colonization of Gram-positive bacteria in the intestinal mucus layer (Supplementary Figure 3 A&B).

Disordered intestinal bacteria in IEC^{ΔChi11} mice contribute to colitis

From the above data, it is evident that Chi3l1 is secreted into the intestinal mucus to influence the colonization of Gram-positive bacteria, particularly *Lactobacillus*. We are now interested in understanding the role of Chi3l1-regulated microbiota in a pathological condition. We observed a significant increase in *Chil1* mRNA expression in the colon tissues of patients with either Crohn's disease (CD) or Ulcerative colitis (UC) compared to normal tissues (Fig. 5A). To investigate further, we created a colitis mouse model by subjecting Villin-cre and IEC^{ΔChi11} mice to a 2% dextran sulfate sodium (DSS) diet for 7 days (Fig. 5B). The severity of colitis was assessed based on weight loss, colon length, and tissue damage. Without the DSS challenge, the colon length and structure were similar between Villin-cre and IEC^{ΔChi11} mice (Fig. 5D & E). However, upon DSS challenge, the IEC^{ΔChi11} mice showed significantly shorter colon length, faster body weight loss, and more severe inflammation compared to the Villin-cre mice (Fig. 5C-E).

To rule out the effects of Chi3l1 on the host contributed to colitis, we pretreated the mice with antibiotics to eliminate gut microbiota before inducing colitis (Supplementary Figure 4A). The Universal bacterial 16S rRNA qPCR data indicated that the majority of gut microbiota were eliminated after antibiotics treatment (Supplementary Figure 4B). However, the IEC^{ΔChi11} mice exhibited a milder colitis phenotype, including slower body weight loss, longer colon length, and less inflammation compared to the Villin-cre mice (Supplementary Figure 4C-E). We believe that this could be due to the relationship between Chi3l1 and inflammation. Based on these findings, it is apparent that Chi3l1's effects on gut microbiota play a more significant role in colitis.

To further elucidate the role of Chi3l1-regulated gut microbiota in colitis, we conducted fecal microbiota transplantation (FMT) and *Lactobacillus reuteri* transplantation experiments. We first eliminated gut microbiota through a 10-day course of antibiotics and then performed FMT from Villin-cre mice or administered oral gavage of *Lactobacillus reuteri* to IEC^{ΔChi11} mice for 2 weeks, followed by a 7-day period of 2% DSS feeding (Fig 5F). FMT partially restored the colon length of IEC^{ΔChi11} mice to that of Villin-cre mice after the DSS challenge, but did not have an impact on body weight loss or the level of inflammation in the gut (Fig. 5G-I). IEC^{ΔChi11} mice transplanted with *Lactobacillus* displayed a similar colitis phenotype as Villin-cre mice, characterized by similar

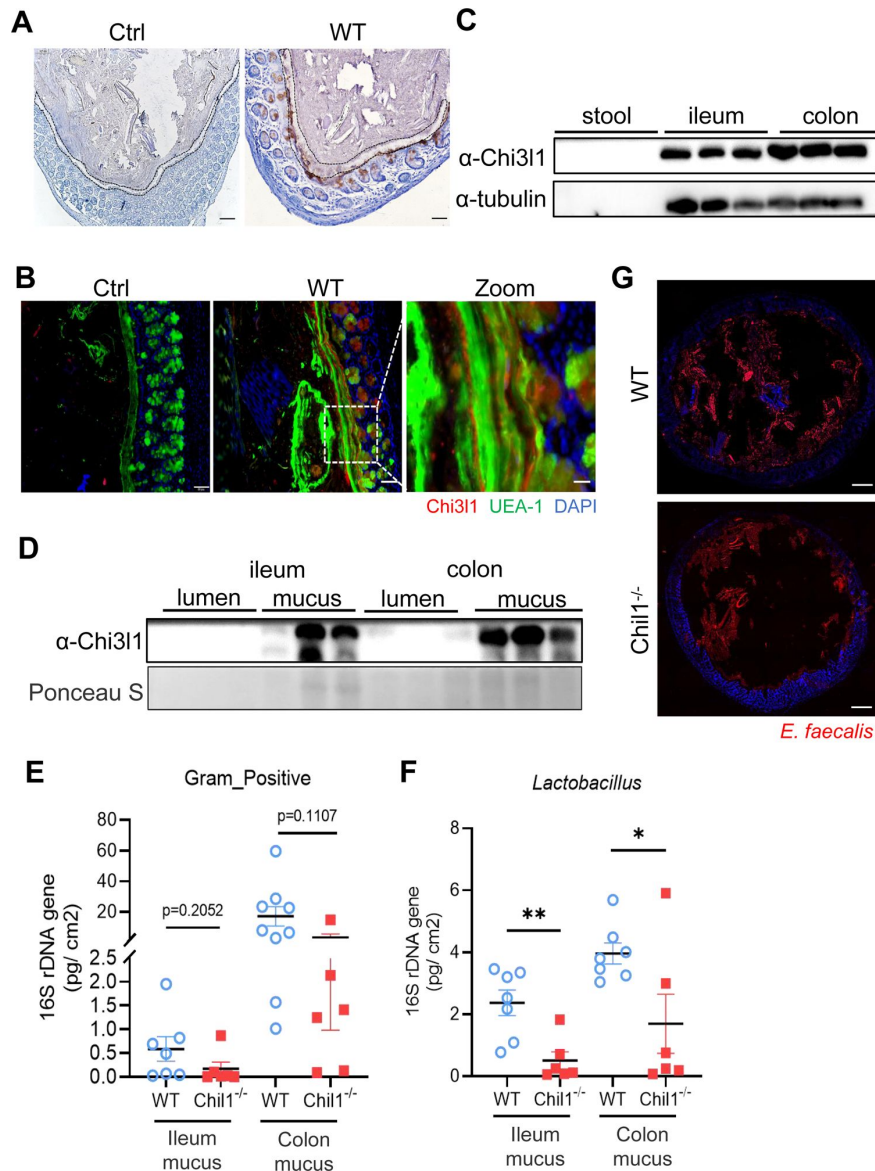


Figure 4.

Chi3l1 promotes the colonization of Gram-positive bacteria in intestinal mucus layer.

(A) IHC staining to detect Chi3l1 in colon mucus layer from wildtype mice. Ctrl (without application of anti-Chi3l1 antibody), WT (wildtype C57B/6J mice). Black dotted line outlines mucus layer. Scale bar, 50 μ m (Ctrl, WT). **(B)** Colons were collected from wildtype mice and stained with UEA-1 (green), Chi3l1 (red), and nuclear DAPI (blue). Ctrl (without application of first antibody), WT (wildtype C57B/6J mice). Scale bars, 50 μ m (Ctrl, WT) and 20 μ m (zoom). **(C)** Stool, ileum and colon tissues were collected from wildtype mice. Western blot was used to detect Chi3l1 expression in these samples. n=3 mice/sample. **(D)** Both luminal and mucus-associated proteins of either ileum or colon were extracted. Western blot was used to detect Chi3l1 expression in these samples. lumen (luminal proteins), mucus (mucus-associated proteins). n=3 mice/sample. **(E&F)** qPCR analysis of specific bacteria in the ileum and colon mucus microbial communities of wildtype and Chi3l1^{-/-} littermates. **(E)** qPCR analysis of Gram-positive bacteria is shown. **(F)** qPCR analysis of Gram-positive bacteria is shown. Values for each bacterial group are expressed relative to total 16S rRNA levels. WT (wildtype C57B/6J mice). Mean $\pm$ SEM is displayed. Two-tailed, unpaired student t-test was performed. P value is as indicated in E. *P<0.05, **P<0.01, n=6-9/group. **(G)** Rectal injection of both wildtype and Chi3l1^{-/-} mice with FDAA-labeled *E. faecalis* (a Gram-positive bacteria strain) for 4 hour. Colon sections were collected and colonization of *E. faecalis* was examined under microscope. Nuclei were stained with DAPI. Representative images are shown in A, B, G, n=3-4 mice/group.

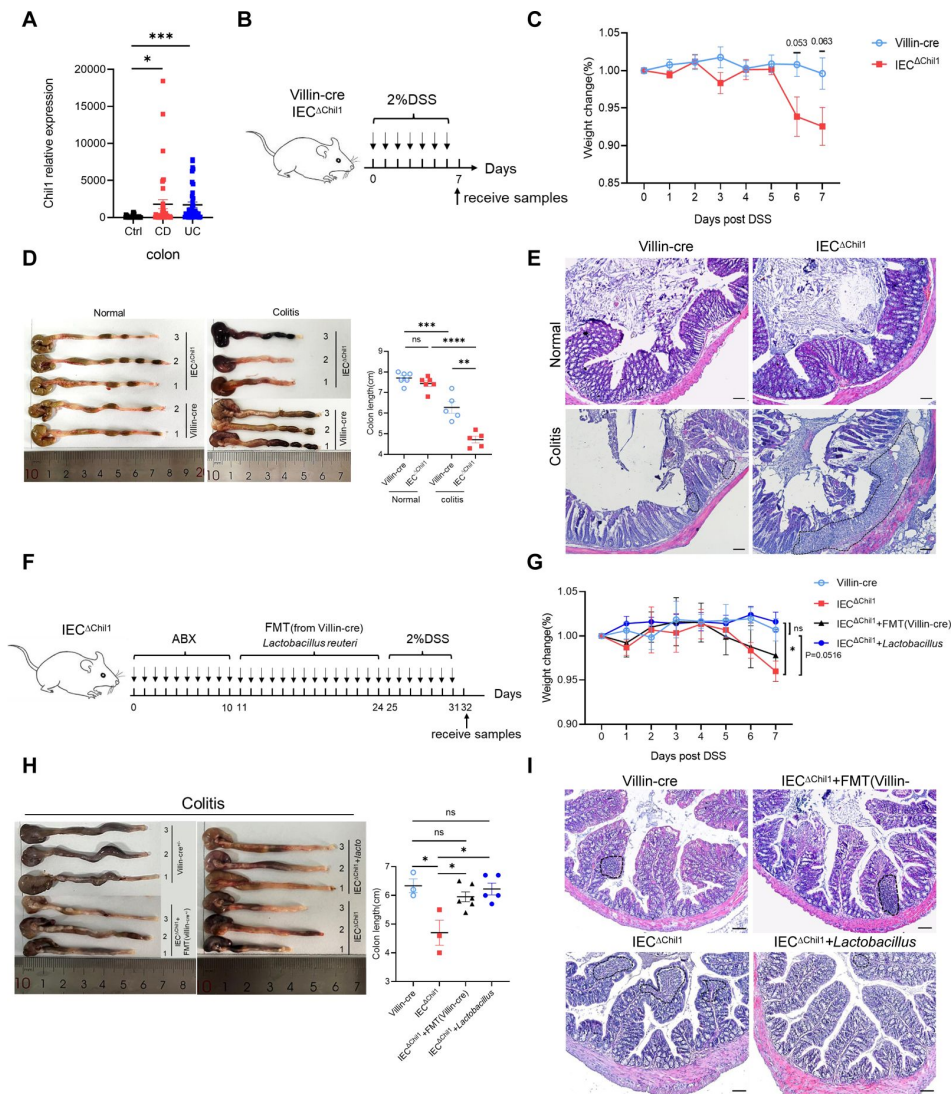


Figure 5.

Disordered intestinal bacteria in IEC Δ Chil1 mice contribute to IBD.

(A) *Chil1* mRNA relative expression in colon tissues of patients without gut disease (controls, n=35) or with Crohn's disease (CD, n=40), ulcerative colitis (UC, n=40), (GEO Datasets: SRP303290), Mann-Whitney test was performed. *P < 0.05; ***P < 0.001. **(B)** Schematic model of the experimental design. Both Villin-cre and IEC Δ Chil1 littermates were fed with 2% DSS in drinking water to induce colitis. **(C)** Weight change of Villin-cre and IEC Δ Chil1 littermates during DSS feeding. Weight change (%) = Current weight/Initial weight. P values are as indicated. **(D)** Representative colonic length from Normal and DSS-treated Villin-cre and IEC Δ Chil1 littermates (left) and the statistics of colonic length (right) (**P < 0.01; ***P < 0.001; ****P < 0.0001, ns, not significant). **(E)** H&E staining of mice colon from Normal and DSS-treated Villin-cre and IEC Δ Chil1 littermates. The inflamed areas are outlined by white dotted line, Scale bars=100 μ m. **(F)** Schematic of the experimental design. First, antibiotics were used to eliminate gut microbiota for 10 days, and then either fecal microbiota from Villin-cre mice (FMT) or *Lactobacillus reuteri* were transplanted back to IEC Δ Chil1 mice orally every day for 2 weeks. Finally, colitis mouse model was constructed by 2% DSS feeding in drinking water for another 7 days. **(G-I)** Villin-cre and IEC Δ Chil1 were only fed with 2% DSS in drinking water for 7 days. IEC Δ Chil1+FMT(Villin-cre), and IEC Δ Chil1+*Lactobacillus* were constructed as described in F. **(G)** Weight change of Villin-cre, IEC Δ Chil1, IEC Δ Chil1+FMT(Villin-cre), and IEC Δ Chil1+*Lactobacillus* mice during DSS feeding. **(H)** Representative colonic length from Villin-cre, IEC Δ Chil1, IEC Δ Chil1+FMT(Villin-cre), and IEC Δ Chil1+*Lactobacillus* mice (left) and the statistics of colonic length (right). Mean $\pm$ SEM is displayed in G, H. one-way ANOVA was performed. P value is as indicated. *P<0.05, ns, not significant, n=3-6/group. **(I)** H&E staining of mice colon from Villin-cre, IEC Δ Chil1, IEC Δ Chil1+FMT(Villin-cre), and IEC Δ Chil1+*Lactobacillus* mice after DSS treatment. The inflamed area is outlined by black dotted line, Scale bars=100 μ m. Representative images are shown in C, E, H, I, n=3-6 mice/group.

weight loss, colon length reduction, and gut inflammation (**Fig. 5G-I**). These findings further validate the notion that Chi3l1-regulated gut microbiota, especially *Lactobacillus*, offers protection against colitis.

Discussion

The intricate relationship between gut bacteria and their host organisms is crucial for health, maintained through co-evolution and co-speciation. Understanding how the host influences these bacterial communities is critical to understand the complex interplay between host and gut bacteria. In this study, we discovered that Chi3l1 interacts with gut microbiota via peptidoglycan, aiding the mucus colonization of beneficial bacteria like *Lactobacillus*, which protects against colitis (**Fig. 6**). Our “scaffold model” demonstrates that Chi3l1 binds to bacterial peptidoglycan, helping anchor and organize bacteria within the mucus layer. This interaction promotes the colonization of beneficial bacteria, enhancing gut health.

Several studies have suggested a potential correlation between Chi3l1 and bacteria. For instance, it has been demonstrated that Chi3l1 is produced by intestinal epithelial cells in enteritis disease and aids in the elimination of pathogenic bacteria in the gut by neutrophils[24]. Another study observed an increase in Chi3l1 expression in mammary tissues of dairy cows infected with pathogenic *E. coli*, which promoted the recruitment of neutrophils[25]. Furthermore, Chi3l1 was found to be induced during *Streptococcus pneumoniae* infection, where it enhanced bacterial elimination by preventing the death of lung macrophages and improving host tolerance[26]. One study demonstrated that Chi3l1 contributes to the pathogenesis of colitis, possibly by facilitating the adhesion and invasion of bacteria onto colonic epithelial cells. However, we observed the presence of the Gram-negative bacterium *Salmonella typhimurium* in the mice used in their study[27]. According to standard for SPF-grade mice, *Salmonella* should not be present in laboratory mice, and it was also not detected by our 16S RNA sequencing. However, if mice are inadvertently infected with *Salmonella* in the context of IBD, it could likely exacerbate the development of IBD. Our data also demonstrate that Chi3l1 can bind to Gram-negative bacteria (**Figure 2B**). Therefore, the role of Chi3l1 on microbiota or IBD is heavily dependent on the environmental context. Another study found that caffeine treatment reduce Chi3l1 RNA expression levels, alleviate colitis, and decrease bacterial translocation[28]. However, previous research suggested that caffeine can directly inhibit inflammasome activation by suppressing MAPK/NF- κ B signaling pathways[29]. Therefore, caffeine treatment may directly reduce colitis by inhibiting inflammation. Moreover, an additional control group of mice with Chi3l1 specifically knocked out in the gut is needed to examine the role of caffeine in the alleviation of colitis. However, the detailed mechanism of the interaction between Chi3l1 and the gut microbiota remains incompletely understood, partly due to the absence of Chi3l1-specific knockout mice and variations in mouse husbandry conditions. Here, by using intestinal Chi3l1-specific knockout mice, we demonstrated a new function of Chi3l1 in gut, which shapes bacterial colonization through direct interaction with bacterial cell wall component, PGN. Moreover, Chi3l1-regulated gut microbiota, especially *Lactobacillus*, offers protection against colitis.

The bacterial cell wall is a complex structure mainly composed of peptidoglycan, but it may also contain other components such as teichoic acids and lipoteichoic acid in gram-positive bacteria, or an outer membrane containing various polysaccharides, lipids, and proteins in gram-negative bacteria[30]. Our findings indicated that lipopolysaccharide (LPS), a component of the bacterial cell wall, can slightly increase Chi3l1 expression (**Fig. 1F & G**), but higher levels of LPS do not further enhance Chi3l1 expression (data not shown). This suggests that there might be other components of the cell wall that can induce Chi3l1 expression in intestinal epithelial cells. Given the structural similarity between chitin and peptidoglycan (**Fig. 2A**), it is likely that Chi3l1 binds to the polysaccharide chains rather than the tetrapeptide in peptidoglycan. Previous studies have investigated the crystal structure of human Chi3l1 in complex with chitin, revealing a binding

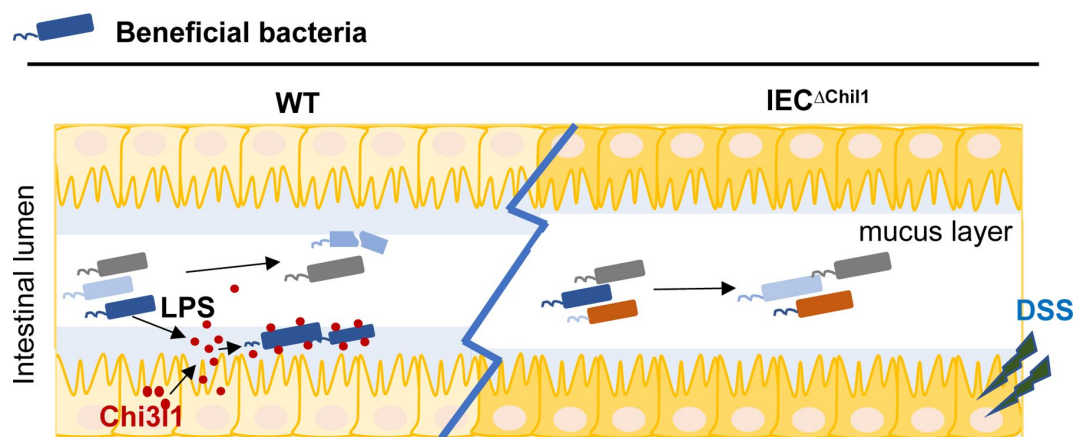


Figure 6.

A schematic working model.

Intestinal epithelial cells are stimulated by the gut microbiota to express Chi3l1. Once expressed, Chi3l1 is secreted into the mucus layer where it interacts with the gut microbiota, specifically through a component of bacterial cell walls called peptidoglycan. This interaction between Chi3l1 and bacteria is beneficial for the colonization of bacteria in the mucus, particularly for gram-positive bacteria like *Lactobacillus*. Moreover, a deficiency of Chi3l1 leads to an imbalance in the gut microbiota, which exacerbates colitis induced by dextran sodium sulfate (DSS).

groove with different subsites for chitin fragments[31]. Other studies have also identified specific amino acids in chitinases that play a key role in the interaction with chitin[32, 33]. Nevertheless, further investigation is necessary to better understand the binding sites in Chi3l1 for peptidoglycan.

In the colon, there are two distinct parts of the mucus layer. The inner layer is attached and has a low number of microbes, while the outer layer is looser and densely populated by microorganisms. [34]. The diversity of bacteria in the mucus layer is similar to that found in the gut lumen[35]. It has to be noted that not all Gram-positive bacteria were reduced in *Chi1^{-/-}* mice, as we found an increase in *Turicibacter* in the colon (Fig. 3G) and feces (data not shown). We believe this may be due to a combined effect of the host and gut microbiota. Another important factor for microbial growth in the colon is the integrity of the mucus barrier[11]. We noticed a thinning of the mucus barrier in the mice lacking Chi3l1 compared to normal mice (Supplementary Figure 3 A&B).

However, we did not observe an increase in mucin-degrading bacteria such as *Bacteroides* or *Allobaculum*[36, 37] or a decrease in mucin-producing cells in these mice. We think that there may be bacteria that aid in the formation of the gut mucus, and these bacteria are decreased in mice lacking Chi3l1. Recent studies have introduced an “encapsulation model” regarding the nature of mucus in the colon. According to this model, the mucus in the proximal colon forms a primary encapsulation barrier around fecal material, while the mucus in the distal colon forms a secondary barrier.[38] Our findings indicate that Chi3l1 is expressed throughout the entire colon, including the proximal, middle, and distal sections (data not shown). This suggests that Chi3l1 likely promotes bacterial colonization across the entire colon. Despite most mucus being expelled with feces, the constant production of mucus and the minimal presence of Chi3l1 in feces (Figure 4C) indicate that Chi3l1 continuously plays a role in promoting the colonization of microbiota.

In patients with inflammatory bowel disease (IBD), the density and diversity of the microbial community in the intestines are reduced. Specifically, there is a decrease in *Firmicutes* and an increase in *Bacteroides* and facultative anaerobic bacteria like *Enterobacteriaceae*[39]. However, the cause of colitis in IBD is still a subject of debate. Our data suggest that disruptions in the gut microbiota contribute to colitis. In summary, our study demonstrates that bacterial challenge induces the expression of Chi3l1 in intestinal epithelial cells. Once produced, Chi3l1 is released into the mucus layer where it interacts with the gut microbiota, particularly through peptidoglycan, a primary component of bacterial cell walls. This interaction is beneficial for the colonization of bacteria, especially gram-positive bacteria like *Lactobacillus*, in the mucus layer. Dysbiosis resulting from a lack of Chi3l1 exacerbates DSS-induced colitis, highlighting the role of dysbiosis as a contributing factor to colitis.

Methods

Animal experiments and procedures

C57BL/6J (Strain No. N000013), Germ-free (Strain No. N000295), *Chi1^{fl/fl}* (Strain No. T013652), *Chi1^{-/-}* (Strain No. T014402) mice were purchased from GemPharmatech. Villin-cre mice were provided by Dr. Qun Lu (Yunnan University, China.). All mouse colonies were maintained at the animal core facility of Yunnan University. C57BL/6J was used as wildtype control since *Chi1^{-/-}* mice are on the C57BL/6J background, as determined by PCR (data not shown). The animal studies described have been approved by the Yunnan University Institutional Animal Care and Use Committee (IACUC, Approval No. YNU20220256). Female mice aged 8-10 weeks old were used in most studies.

Genotyping Tail clippings were placed in 1.5 ml tubes with 75 μ L of master mix solution (60 μ L H₂O, 7.5 μ L 250 mM NaOH, 7.5 μ L 2 mM EDTA) and incubated at 98°C for 1 hour. After cooling to 15°C, 75 μ L of neutralization buffer (40 mM Tris HCl, pH 5.5) was added, and the samples were centrifuged at 4000 rpm for 3 minutes. A 1:10 dilution was prepared by mixing 2 μ L of supernatant with 18 μ L of water for genotyping PCR. A 25 μ L PCR reaction mix was prepared with 12.5 μ L of 2x Taq Master Mix (Dye Plus, Vazyme P112-03), 1 μ L of each primer, 2 μ L of template, and water. PCR was conducted using a Biorad machine with the following program: 95°C for 5 minutes; 98°C for 30 seconds; 65°C with a 0.5°C decrement per cycle for 30 seconds; 72°C for 45 seconds, repeating steps 2-4 for 20 cycles; 98°C for 30 seconds; 55°C for 30 seconds; 72°C for 45 seconds, repeating steps 5-7 for 20 cycles; 72°C for 5 minutes; and hold at 10°C. Primer sequences are listed in Table S1.

Rectal administration of FDAA-labeled *E. faecalis* For FDAA-labeled *E. faecalis*, *E. faecalis* were grown in LB media until reaching mid-exponential phase, approximately 4 hours. FDAA was then added to the culture media to a final concentration of about 17 μ M. The *E. faecalis* continued to grow for 4 hours and were then harvested by centrifugation (5000 x g for 10 min), washed, and resuspended in PBS at a density of 5×10^9 CFU/ml. For rectal administration, wildtype and *Chil1*^{-/-} mice were fasted overnight (5:00pm to 9:00am) before intraperitoneally (i.p.) injecting them with 400mg/kg tribromoethanol (Nanjing AIBI BioTechnology, M2910), followed by rectal injection of 1×10^9 FDAA-labeled *E. faecalis* in 200 μ L PBS via a flexible catheter. The catheter was inserted into the anus to a depth of 4.5 cm, and the *E. faecalis* were injected slowly to avoid overflow. The mice were then kept upside down for approximately 2 minutes. After 4 hours of rectal injection, the mice's colons were collected and immediately embedded in OTC embedding medium for observation on 5 μ m thick frozen sections. OP50-mCherry (1×10^9 CFU/mice) treated same as *E. faecalis*.

DSS-induced mouse colitis Villin-cre and IEC ^{Δ Chil1} mice were fed a 2% dextran sodium sulfate (DSS, MP, 9011-18-1) solution in their drinking water for 7 days. The mice's body weight was monitored daily during the feeding period. After the 7-day DSS treatment, the mice were sacrificed. Colons were collected, and their length was measured from the cecum to the rectum. Colon paraffin sections were harvested, and H&E staining was performed to examine gut inflammation.

Antibiotics treatment IEC ^{Δ Chil1} mice were fed an antibiotics mixture containing 0.5 mg/ml of Metronidazole (Solarbio, M8060), 1 mg/ml of vancomycin (Solarbio, V8050), 1 mg/ml of Ampicillin (Solarbio, A8180), and 0.5 mg/ml of Neomycin Sulfate (Solarbio, N8090) in their drinking water for 7 days (approximately 5ml per mouse per day). After the 7-day antibiotics treatment, the mice were orally gavaged with 200 μ L of the antibiotics mixture for another 3 days. Microbiota depletion was examined in feces using 16S rRNA qPCR on the 10th day of antibiotics feeding.

Fecal microbiota transplantation (FMT) Fresh feces were collected from 8-10 weeks old Villin-cre mice and immediately snap-frozen in liquid nitrogen. On the experimental day, feces were resuspended in PBS to a concentration of 200 mg/ml and centrifuged at 350g for 5 minutes to collect the supernatant. Antibiotics pre-treated IEC ^{Δ Chil1} mice were orally gavaged with 10 μ L of the dissolved feces per gram of mouse weight for 14 days.

Oral gavage of *Lactobacillus reuteri* *Lactobacillus reuteri* was grown in MRS broth at 37°C for 48 hours under anaerobic conditions. The bacteria were harvested by centrifugation (5000 x g for 10 min), washed, and resuspended in PBS at a density of OD₆₀₅=1.2-1.3/ml. Antibiotics pre-treated IEC ^{Δ Chil1} mice were orally gavaged with 200 μ L of the dissolved *Lactobacillus reuteri* per mouse for 14 days.

Bacterial colony identification

Fresh feces were collected from 8-week-old wild-type mice and dissolved in LB culture medium. The dissolved feces were then cultured at 37°C for 12 hours and plated onto LB agar plates. The grown colonies were picked using sterile pipette tips and resuspended in 20 μ L of sterile water. A

Aim genes	PCR No.	Primer No.	Sequence	Band Size
floxed	① 5'arm (floxed)	F1(JS05000-Chil1-5wt-tF1)	CTTGTTTCAGCCAAG GTGATGGGTA	WT:270bp targeted:375bp
		R1(JS05000-Chil1-5wt-tR1)	CTACCTGATTGCTG GGGCTCATT	
	② 3'arm	F2(JS15000-Chil1-3wt-F1)	CCAGTATTTAGAGG CAGAGAGATGGTG	WT:283bp targeted:384bp
		R2(JS15000-Chil1-3wt-R1)	CTCGAATTCAGAAA TCTGCCTGCCT	
Chil1 ^{-/-}	①	F1(JS05000-Chil1-5wt-tF1)	CTTGTTTCAGCCAAG GTGATGGGTA	WT: 2960 bp Targeted: ~256 bp
		R1(JS05000-Chil1-3wt-tR1)	CTCGAATTCAGAAA TCTGCCTGCCT	
	②	F2(JS15000-Chil1-wt-F1)	CTGTTAGTTGCACC TTGGAGCAGTCA	WT: 309 bp Targeted:0 bp
		R2(JS15000-Chil1-wt-R1)	CAGATATAGGAGA ACATCCAGTCTGGG	
Chil1-reporter	①	EGE-YQH-063-A-WT-F	CAGTTTCCCAACCC CTCACCTC	Targeted:485 ~bp
		EGE-YQH-063-A-Mut-R	AAGAAGTCGTGCTG CTTCATGTGGT	
	②	EGE-YQH-063-A-WT-F	CAGTTTCCCAACCC CTCACCTC	WT:323 Targeted:1106~ bp
		EGE-YQH-063-A-WT-R	CTACCCTAGTTCCTT GTTCTCCA	
Cre	Cre①	Cre1	AGGTTCGTTCACCTC ATGGA	Cre:250bp Internal Control (Fabpi)=480bp
		Cre2	TTCGACCAGTTTAG TTACCC	
	IntC500②	IntC500F	CCTCCGGAGAGCAG CGATTAAAAGTGTC AG	
		IntC500R	TAGAGCTTTGCCAC ATCACAGGTCATT AG	

Table S1

PCR reaction was performed using 2 μ l of the bacterial suspension as template DNA and universal bacterial 16S rRNA primers (27F, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R, 5'-GGTTACCTTGTACGACTT-3') with reaction conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30sec, 55°C for 30sec, 72°C for 2min and then 72°C for 20min. The amplicons were then sequenced, and the resulting sequences were analyzed using BLASTN and the NCBI database for species identification.

Treatment of DLD-1 cells with live, heat-killed bacteria or LPS

Bacteria harvested from mouse feces or specific strains were grown in LB medium at 37°C for 12 hours under aerobic conditions. The bacteria were then collected by centrifugation (5000 $\times$ g for 10 min), washed, and resuspended in PBS at a density of 1.2×10^{10} CFU/ml. Live bacteria were used directly, while heat-killed bacteria required further heating at 80°C for 30 minutes. DLD-1 cells were grown in DMEM medium supplemented with 10% FBS. Prior to treatment, the cells were replated and allowed to reach 80% confluency. Either live or heat-killed bacteria were added to the cells at a multiplicity of infection of 20 each well, and 100 μ g/ml LPS treatment (Sigma, L4392; diluted in PBS) was also performed. After 12-hour incubation, cellular proteins were extracted for western blot analysis or the cells were subjected to immunofluorescent staining.

Cell Culture and Bacterial Adhesion Assay

DLD-1 cells were cultured in 6-well plates until they reached approximately 80% confluency per well. The cells were then washed three times with PBS to remove any residual antibiotics. Fresh DMEM supplemented with 10% FBS (without antibiotics) was added to the cells. *E. coli* strains were introduced to each well at a multiplicity of infection (MOI) of 20 and incubated for 2.5 hours at 37°C. After incubation, the cells were washed three times with PBS. For the adhesion assays, the cells were lysed with 1 mL of 1% Triton-X100 (BBI, 9002-93-1) in deionized water for 30 minutes. The cell lysates were then plated onto Luria broth (LB) agar plates at various dilutions and incubated overnight at 37°C. The following day, colonies were counted and calculated as colony-forming units (CFU)/mL.

shRNA Lentivirus Packaging and Transfection

For the packaging of shRNA lentivirus, shRNA control and Chi3l1 shRNA1 lentiviruses were packaged in 293T cells. The cell culture medium containing the virus was collected and stored at -80°C. For transfection of DLD-1 cells, the cells were cultured in 6-well plates until they reached approximately 80% confluency. The cell culture medium was then replaced with a mixture of culture medium containing the virus and fresh cell culture medium in a 1:1 ratio. Following transfection, the bacterial adhesion assay was performed as described above.

Bacterial or peptidoglycan binding assay

Different bacteria strains were grown in LB medium at 37°C for 12 hours under aerobic conditions. The bacteria were collected by centrifugation (5000 $\times$ g for 10 min), washed, and resuspended in MES buffer (25mM MES, 25mM NaCl, Ph=6.0) at a density of 5×10^9 CFU/ml. 1 μ g Recombinant mouse Chi3l1 (rmChi3l1) was added to the bacterial suspension and incubated at 4°C under rotation overnight. Supernatant, wash fractions, and bacterial-bound fractions were collected and analyzed using western blot analysis. For the peptidoglycan binding assay, 1 μ g rmChi3l1 or recombinant human Chi3l1 (rhChi3l1) and bovine serum albumin (BSA) was incubated with 100 μ g peptidoglycan (PGN). The incubation and wash procedure were similar to the bacterial binding assay, and the proteins in each fraction were analyzed using silver staining or western blot.

Hematoxylin/eosin(H&E) and Periodic acid-Schiff and Alcian blue (AB-PAS) staining

Tissues were fixed with buffered 10% paraformaldehyde (BI, E672001-0500) overnight at 4°C and embedded in paraffin. Ultra-thin tissue slices (5µm) were prepared and deparaffinized. H&E staining was performed on the tissue sections, and slides were examined under a microscope (Leica). For AB-PAS staining, tissues were fixed in Carnoy's solution (60% Ethanol, 30% Chloroform, 10% Acetic Acid) for 24 hour at 4°C and embedded in paraffin. AB-PAS staining (Solarbio, G1285) was performed according to the manufacturer's protocol. The staining was visualized under the microscope (Leica).

Immunohistochemical (IHC) and immunofluorescent (IF) staining

Tissue paraffin sections were prepared as previously described for H&E staining. Antigen retrieval was performed by treating the sections with citric acid (pH 6.0) at 95°C for 15 minutes, followed by cooling to room temperature. The sections were then washed with PBS and ddH₂O. To block any nonspecific binding, a blocking buffer containing 5% goat serum, 3% BSA, and 0.1% Triton X-100 in PBS was applied to the sections for 1 hour at room temperature in a humidity chamber. The sections were then incubated with anti-Chi3l1 primary antibodies (Invitrogen, PA5-95897, 1:200), followed by staining with Goat anti-Rabbit IgG (H+L) Secondary Antibody-Biotin (Invitrogen, 65-614, 1:2000). Finally, the sections were stained with Horseradish Peroxidase conjugate antibody (Invitrogen, A2664, 1:2000) and developed with DAB for 10 minutes. The slides were examined under a microscope (Leica).

Immunofluorescent staining was also performed on paraffin sections using specific primary antibodies. These included anti-MUC2 (Invitrogen, PA5-103083, 1:50), anti-Chi3l1 (Abcam, ab180569, 1:400; antigen retrieval with Tris-EDTA at pH 9.0), anti-ChgA (Santa Cruz, sc-393941, 1:200; antigen retrieval with citrate at pH 6.0), anti-UEA-1-FITC (GeneTeX, GTX0151, 1:200; antigen retrieval with citrate at pH 6.0), and anti-LTA (Invitrogen, MA1-7402, 1:50). The secondary antibodies used were 488-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (Jackson ImmunoResearch, 111-545-003, 1:1000) and 594-conjugated Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, 115-585-003, 1:1000). Slides were washed and mounted with antifade medium (Vectashield, H-1000-10). Nuclei were stained with DAPI (Beyotime, c1006, prediluted). Images were captured using a fluorescence microscope (Leica, 2084 DP-80).

Immunofluorescent staining on DLD-1 cells, cells were seeded on coverslips in 12-well plate and challenged with 100 pg/ml LPS (Sigma, L4391; diluted in PBS) for 12 hours. Cells were washed with cold-PBS twice gently, then fixed with 2% paraformaldehyde in PBS at room temperature for 10 min. After removal of fixation buffer and wash twice with cold-PBS, cells were blocked with blocking buffer (3% BSA, 0.5% Triton-X-100 in PBS) for 1 hour in humidity chamber at room temperature. Rabbit anti-Chi3l1 antibody (Proteintech, 12036-1-AP; 1:200) was applied at room temperature for 1 hour. After wash with 1 x TBST three times, secondary antibody AlexaFluor 488 (Jackson ImmunoResearch, 111-545-003, 1:1000) was applied for another 1 hour at room temperature in a humidified chamber under darkness. Finally, cells were counterstained with DAPI (beyotime, C1006) and mounted onto slides. Images were captured using an Olympus BX53F2 microscope.

Fluorescence in situ hybridization (FISH)

Murine intestinal paraffin sections were prepared according to the previously described method for H&E staining. The tissues sections were rehydrated using a graded ethanol series and then washed with distilled water. The gram-positive bacterial probe, consisting of three different sequences (/5Alex550N/TGGAAGATTCCCTACTGC/3AlexF550N/, /5Alex550N/CGGAAGATTCCCTACTGC/3AlexF550N/,

/5Alex550N/CCGAAGATTCCCTACTGC/3AlexF550N/), or the control nonspecific probe (/5Alex550N/ACTCCTACGGGAGGCAGC/3AlexF550N/), was diluted to a concentration of 100 nM in FISH hybridization buffer (containing 20 mM Tris pH 7.2, 0.9 M NaCl, and 0.1% SDS) and applied to the slides. The slides were then incubated overnight at 56°C in a humidified chamber. Following incubation, the slides were washed and the nuclei were counterstained with DAPI. The images were captured using a fluorescence microscope (Leica, 2084 DP-80).

Immunoblot and silver staining

Protein extraction from cultured cells involved lysing the cells in 2% SDS lysis buffer, which is prepared by dissolving 2g of SDS powder in 100ml of sterilized ddH₂O. Bacteria or peptidoglycan precipitates were resuspended in MES buffer, which contained 25mM MES, 25mM NaCl, and had a pH of 6.0. For protein extraction from mice ileum and colon tissues, 30mg of snap-frozen tissues were homogenized in 1ml of RIPA buffer, which contained 10mM Tris-HCl (pH 8.0), 1mM EDTA, 0.5mM EGTA, 1% TritonX-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl, 1mM PMSF, and a proteinase inhibitor. The lysates were then supplemented with 5x SDS loading buffer to a final concentration of 1x. The resulting mixture was subsequently boiled at 100°C for 10 minutes and centrifuged at 4°C and 12,000 rpm for 10 minutes. The supernatants were collected for western blot analysis. The supernatants were separated using a 10% SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat milk in TBST buffer (containing 0.1% Tween-20 in tris-buffered saline) and sequentially incubated with primary antibodies and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were detected using enhanced chemiluminescence (ECL) reagent with a Minichemi Chemiluminescence Imaging System. The primary antibodies used included anti-Chi311 (RD, MAB2649, 1:2000) and anti-alpha-Actinin (Cell Signaling, 69758S, 1:1000). The secondary antibodies used were goat anti-Rat-IgG (Cell Signaling, 7077s, 1:1000) and goat anti-mouse (Invitrogen, 62-6520, 1:1000). For silver staining, the PAGE gel was subjected to silver staining using a fast-silver staining kit (Beyotime, P0017S) following the manufacturer's instructions.

DNA extraction for 16S rRNA analysis

For isolation of luminal contents from murine ileum and colon, a 9 cm section of ileum and 3 cm section of colon were cut open longitudinally, and luminal contents were scratched off into a pre-weighed 2-ml sterile freezing vial. The weight of the contents was recorded for further processing. For isolation of mucus from murine ileum and colon, tissues were flushed with 2 ml of ice-cold PBS into a pre-weighed 2-ml sterile freezing vial after removal of the luminal contents. The mucus was then pelleted by centrifugation at 10,000g for 10 minutes, and the supernatant was removed. For feces isolation, fresh murine feces were collected into a sterile freezing vial weighing 2 ml, and immediately snap-frozen in liquid nitrogen. For bacterial DNA extraction, DNA was extracted and purified following the manufacture's protocol using the EIANamp stool DNA kit (TIANGEN, DP328-02).

Microbiota 16S rRNA gene sequencing

Fecal samples, ileum contents, and colon contents were collected from wildtype, *Chil1*^{-/-} littermates, or Villin-cre, IEC^{ΔChil1} littermates and immediately frozen in liquid nitrogen. The microbial genomic DNA was extracted and 16S rRNA sequencing was performed by Biomarker Technologies. The hypervariable regions V3 and V4 of the bacterial 16S rRNA gene were sequenced using universal primers that flank these regions V3 (338F 5'-ACTCCTACGGGAGGCAGCA-3') and V4 (806R 5'-GGACTACHVGGGTWTCTAAT-3'). The sequencing was done using the Illumina Sequencing platform. The resulting 16S rRNA gene sequences were analyzed using scripts from the BMK Cloud platform (www.biocloud.net). The microbial classification was performed using the SILVA138 and hierarchical clustering algorithms. The OTUs were determined by clustering the sequences with 97% similarity and were classified into different taxonomic ranks. The relative

abundance of each bacterial species was visualized using R software. The raw 16S rRNA gene sequencing data can be accessed on the BMKCloud platform under the project ID Microbial_updateReport_20211221092022313.

16S qPCR analysis

Quantitative PCR was performed using SYBR green master mix (Thermo Fisher, A25742) in triplicates. This was done on a Real-Time PCR QuatStudio1 with accompanying software, following the instructions provided by the manufacturer (Life Technologies, Grand Island, NY, USA). The abundance of specific bacterial groups in the intestine was determined using qPCR with either universal or bacteria-specific 16S rRNA gene primers. Standard curves were constructed with *E.Coli OP50* 16S rRNA gene, which was amplified using conserved 16S rRNA primers. It should be noted that qPCR measures the number of 16S gene copies per sample, not the actual bacterial numbers or colony forming units. Primer sequences are provided in table S2.

Table S2

qPCR primers

Targeted bacteria	Primer No.	Sequence
Universal	27F	AGAGTTTGATCCTGGCTCAG
	1492R	GGTTACCTTGTTACGACTT
Gram-positive	928F-firm	TGAAACTYAAGGAATTGACG
	1040-firmR	ACCATGCACCACCTGTC
Lactobacillus	F	TGGAAACAGGTGCTAATACCG
	R	GTCCATTGTGGAAGATTCCC

Key source tables

Antibodies and chemicals

Reagent or Antibodies	Source	Identifier
Dextran Sulfate Sodium Salt	MP	CAS:9011-18-1
AB-PAS staining kit	Solarbio	G1285
EIANamp stool DNA kit	TIANGEN	DP328-02
PAGE Gel Fast Preparation Kit	Shanghai Epizyme biotechnology	PG113

gel extraction kit	Omega--	D2500-02
SYBR Green kit	Thermo Fisher	A25742
hematoxylin	Servicebio	G1004
eosin	Biosharp	BL703b
DAB Substrate Kit	ZSGB-BIO	zli-9018
Tris	Solarbio	77-86-1
Sodium chloride	Solarbio	7647-14-5
disodium salt dihydrate (EDTA)	Sangon Biotech	6381-92-6
Sodium dodecyl sulfate (SDS)	BBI	A601336-0500
egtazic acid,Glycol ether diamine	BBI	67-42-5
tetraacetic acid (EGTA)		
TritonX-100	BBI	9002-93-1
Citric acid	Sangon Biotech	77-92-9
Vancomycin	Solarbio	V8050
Ampicillin- Sodium salt	Solarbio	A8180)
Metronidazole	Solarbio	M8060
Neomyein Sulfate	Solarbio	N8090
MRS broth	Solarbio	M8540
Goat serum	Solarbio	
Anti-Chi311 Polyclonal Antibody	Invitrogen	PA5-95897
Cha-A antibody	Santa Cruz	sc-393941
anti-mouse Chi311 Purified Rat	R&D	MAB2649
Monoclonal IgG		
MUC2 Polyclonal Antibody	Invitrogen	PA5-103083
Gram Positive Bacteria LTA	Invitrogen	MA1-7402
Monoclonal Antibody (G43J)		
488-conjugated Affinipure Goat	Jackson	111-545-003
Anti-Rabbit		
immunoresearch Alexa Fluor	Jackson	115-585-003
594 AffiniPure Goat Anti-Mouse		
IgG (H+L)		
Goat anti-Rabbit IgG (H+L)	Invitrogen	65-6140
Secondary Antibody-Biotin		
Horseradish Peroxidase conjugate	Invitrogen	A2664
antibody		
Rabbit anti-Chi311 antibody	Proteintech	12036-1-AP
Goat anti-Rabbit IgG	Jackson ImmunoResearch	111-035-0030
Mouse anti- α -tubulin antibody	sigma	T5168
Goat anti-mouse IgG	Jackson ImmunoResearch	115-035-003
Goat anti-Rat IgG	Jackson ImmunoResearch	112-035-003
DAPI	Beyotime	c1006
Antifade mounting medium	Vectashield	H-1000-10
FDA	5TAMRA; CHINESE PEPTIDE	CS-11-00433
gram-positive bacteria 16S rRNA	Guangzhou Exon Biotech	
probe		

Cell lines/ Bacteria strains

DLD-1 cells	a gift from Dr. Sun Jianwei at Yunnan University	
<i>Staphylococcus saprophyticus</i>	ATCC	15305
<i>E. faecalis</i>	ATCC	33186
<i>Lactobacillus reuteri</i>	ATCC	23272
<i>E. coli</i> strain K12	Dharmacon	Cat #OEC5042

<i>E. coli</i> strain OP50	CGC	RRID:WB STRAIN:OP50
<i>E. coli</i> strain OP50-mCherry	Provided by Bin Qi Lab	[40]
<i>Staphylococcus Sciuri</i>	identified from C57BL/6J	
	wild-type mice stools	
<i>E. coli</i>	identified from C57BL/6J	
	wild-type mice stools	

Statistical analysis

Data were presented as mean $\pm$ SEM. Statistical analyses were carried out using GraphPad Prism (GraphPad Software). Comparisons between two groups were carried out using unpaired Student t test. Comparisons among multiple groups ($n \geq 3$) were carried out using one-way ANOVA. P values are as labeled and less than 0.05 was considered not significant. Patient data were analyzed by Mann-Whitney test.

Author Contributions

Y.C. and R.Z.Y. performed experiments, analyzed data, and wrote the manuscript. B.Q. organized and designed the study. Z.S. initiated, organized, designed the study and wrote the manuscript.

Acknowledgements

We thank Qun Lu (Yunnan University) for providing Villin-cre mice. We thank Wenxiang Fu (Yunnan University) for imaging technical support. We thank Jianwei Sun (Yunnan University) for providing DLD-1 cell line. We thank Zehan Hu (Shanghai Jiao Tong University) for methods of bacteria 16S FISH staining. This work was supported by Ministry of Science and Technology of China (2019YFA0803100, 2019YFA0802100 to B.Q.), National Natural Science Foundation of China (32071129 to Z.S., 32170794 to B.Q.), Yunnan Fundamental Research Projects (202101AT070022, 202001AW070006 to Z.S., 202201AT070196 to B.Q.), Science and Technological Talent Cultivation Plan of Yunnan Province (C619300A086 to Z.S., K264202230211 to B.Q.), Yunnan Provincial Science and Technology Project at Southwest United Graduate School (202302AP370005 to B.Q.)

Declaration of interests

The authors declare no competing interests.

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Icahn School of Medicine at Mount Sinai, New York, United States of America

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Reviewer #1 (Public review):

The manuscript by Chen et al. investigated the interaction between CHI3L1, a chitinase-like protein in the 18 glycosyl hydrolase family, and gut bacteria in the mucosal layers. The authors provided evidence to document the direct interaction between CHI3L1 and peptidoglycan, a major component of bacterial cell wall. Doing so, Chi3l1 produced by gut epithelial cells regulates the balance of gut microbiome and diminishes DSS-induced colitis, potentially through the colonization of protective gram-positive bacteria such as lactobacillus.

The study is the first to systemically document the interactions between Chi3L1 and microbiome. Convincing data were shown to characterize the imbalance of gram-positive bacteria in the newly generated gut epithelial-specific Chi3L1 deficient mice. Comprehensive FMT experiments were performed to demonstrate the contributions of gut microbiome using the mouse colitis model. The manuscript is strengthened by additional mechanistic studies concerning the binding between Chi3L1 and peptidoglycan, and discussions on existing body of literature demonstrating that detrimental roles of Chi3L1 in mouse IBD model, which conflict with the current study.

<https://doi.org/10.7554/eLife.92994.2.sa2>

Reviewer #2 (Public review):

Chen et al. investigated the regulatory mechanism of bacterial colonization in the intestinal mucus layer in mice and its implications to intestinal diseases. They demonstrated that Chi3L1 is a protein produced and secreted by intestinal epithelial cells into the mucus layer upon response to the gut microbiota, which has a turnover effect on facilitating the colonization of gram-positive bacteria in the mucosa. The data also indicate that Chi3L1 interacts with the peptidoglycan of the bacteria cell wall, supporting the colonization of beneficial bacteria strains such as *Lactobacillus*, and that deficiency in Chi3L1 predisposes mice to colitis. The inclusion of a small but pertinent piece of human data added to solidify their findings in mice.

Overall, the experiments were appropriately designed and executed with precision. The revised manuscript represents a significant improvement over the initial version. The inclusion of new, higher-resolution images provides stronger support for the conclusions drawn. Additionally, statistical analyses of the imaging data, as recommended, have been integrated. The authors have effectively addressed the majority of the reviewers' suggestions and criticisms, making this version well-suited for publication.

<https://doi.org/10.7554/eLife.92994.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer 1:

(1) In Figure 1, it is curious that the authors only chose E.coli and staphylococcus sciuri to test the induction of Chi3L1. What about other bacteria? Why does only E.coli but not staphylococcus sciuri induce chi3L1 production? It does not prove that the gut microbiome induces the expression of Chi3L1. If it is the effect of LPS, does it trigger a cell death response or inflammatory responses that are known to induce chi3L1 production? What is the role of peptidoglycan in this experiment? Also, it is recommended to change WT to SPF in the figure and text, as no genetic manipulation was involved in this figure.

Thank you for your valuable feedback and insightful suggestions. In our study, we tried to identify bacteria from murine gut contents and feces using 16S sequencing. However, only *E. coli* and *Staphylococcus sciuri* were identified (Figure 1D). Consequently, our experiments were limited to these two bacterial strains. While we have not tested other bacteria, our data suggest that not all bacteria can induce the expression of Chi3L1. Given that *E. coli* is Gram-

negative and *Staphylococcus sciuri* is Gram-positive, we hypothesized that the difference in their ability to induce Chi3l1 expression might be due to variations between Gram-negative and Gram-positive bacteria, such as the presence of lipopolysaccharides (LPS).

To test this hypothesis, we used LPS to induce Chi3l1 expression. Consistent with our hypothesis, LPS successfully induced Chi3l1 expression (Figure 1F&G). Additionally, we observed that Chi3l1 expression is significantly upregulated in specific pathogen-free (SPF) mice compared to germ-free mice (Figure 1A), demonstrating that the gut microbiome induces the expression of Chi3l1.

Although we have not examined cell death or inflammatory responses, the protective role of Chi3l1 shown in Figure 5 suggests that any such responses would be mild and negligible. Regarding the role of peptidoglycan in the induction of Chi3l1 expression in DLD-1 cells, we have not yet explored this aspect. However, we agree with your suggestion that it would be worthwhile to investigate this in future experiments.

We have also made the suggested modifications to the labeling (Figure 1A) and the clarification in the revised manuscript accordingly (page 3, Line 95-96; Line 102-106).

Thank you again for your constructive feedback.

(2) In Figure 2, the binding between Chi3l1 and PGN needs better characterization, regarding the affinity and how it compares with the binding between Chi3l1 and chitin. More importantly, it is unclear how this interaction could facilitate the colonization of gram-positive bacteria.

Thank you for your insightful suggestions and we have performed the suggested experiments and included the results in the revised manuscript (Figure 2E-G, page 3-4, Line 132-146).

Our results indicate that Chi3l1 interact with PGN in a dose-increase manner (Figure 2E). In contrast, the binding between Chi3l1 and chitin did not exhibit dose dependency (Figure 2E). These findings suggest a specific and distinct binding mechanism for Chi3l1 with PGN compared to chitin.

We conducted DLD-1 cell-bacteria adhesion experiments, using GlmM mutant (PGN synthesis mutant) and K12 (wild-type) bacteria to test their adhesion capabilities. The results showed that the adhesion ability of the GlmM mutant to cells significantly decreased (Figure 2F). Additionally, after knocking down Chi3l1 in DLD-1 cells, we observed a decreased bacterial adhesion (Figure 2G). These findings suggest that Chi3l1 and PGN interaction plays a crucial role in bacterial adhesion.

(3) In Figure 3, the abundance of furmicutes and other gram-positive species is lower in the knockout mice. What is the rationale for choosing lactobacillus in the following transfer experiments?

We appreciate your thorough review. Among the Gram-positive bacteria that we have sequenced and analyzed, *Lactobacillus* occupies the largest proportion. Given the significant presence and established benefits of *Lactobacillus*, we chose it for the subsequent transfer experiments to leverage its known properties and availability, thereby ensuring the robustness and reproducibility of our findings. This is supported by the study referenced below.

Lamas B, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, Bridonneau C, Jegou S, Hoffmann TW, Natividad JM, Brot L, Taleb S, Couturier-Maillard A, Nion-Larmurier I, Merabtene F, Seksik P, Bourrier A, Cosnes J, Ryffel B, Beaugerie L, Launay JM, Langella P, Xavier RJ, Sokol H. CARD9 impacts colitis by altering gut microbiota metabolism of

tryptophan into aryl hydrocarbon receptor ligands. Nat Med. 2016 Jun;22(6):598-605. doi: 10.1038/nm.4102. Epub 2016 May 9. PMID: 27158904; PMCID: PMC5087285.

(4) FDAA-labeled E. faecalis colonization is decreased in the knockouts. Is it specific for E. faecalis, or it is generally true for all gram-positive bacteria? What about the colonization of gram-negative bacteria?

Thank you for your insightful suggestions and we have investigated the colonization of gram-negative bacteria, OP50-mcherry (a strain of *E. coli* that express mCherry) and included the results in the updated manuscript (Supplementary Figure 3B, page 5, Line 197-200). We performed rectal injection of both wildtype and *Chi11*^{-/-} mice with mCherry-OP50, and found that *Chi11*^{-/-} mice had much higher colonization of *E. coli* compared to wildtype mice.

(5) In Figure 5, the fact that FMT did not completely rescue the phenotype may point to the role of host cells in the processes. The reason that lactobacillus transfer did completely rescue the phenotypes could be due to the overwhelming protective role of lactobacillus itself, as the experiments were missing villin-cre mice transferred with lactobacillus.

Thank you for your valuable feedback and thorough review. In our study, pretreatment with antibiotics in mice to eliminate gut microbiota demonstrated that *IECΔChil1* mice exhibited a milder colitis phenotype (Supplementary Figure 4). This suggests that *Chi3l1*-expressing host cells are likely to play a detrimental role in colitis. Consequently, the failure of FMT to completely rescue the phenotype is likely due to the incomplete preservation of bacteria in the feces during the transfer experiment.

We agree with your assessment of the protective role of *lactobacillus*. This also explains the significant difference in colitis phenotype between Villin-cre and *IECΔChil1* mice (Figure 5B-E), as *lactobacillus* levels are significantly lower in *IECΔChil1* mice (Figure 4F). Given the severity of colitis in Villin-cre mice at 7 days post-DSS, even if *lactobacillus* were transferred back to these mice, it is unlikely to result in a significant improvement.

(6) Conflicting literature demonstrating the detrimental roles of Chi3l1 in mouse IBD model needs to be acknowledged and discussed.

Thank you for your insightful suggestions and we have included additional discussions in the revised manuscript (page 6-7, Line 258-274).

Reviewer #2 (Public Review):

(1) Images are of great quality but lack proper quantification and statistical analysis. Statements such as "substantial increase of Chi3l1 expression in SPF mice" (Fig. 1A), "reduced levels of Firmicutes in the colon lumen of IEC Δ Chil1" (Fig. 3F), "Chi11^{-/-} had much lower colonization of E. faecalis" (Fig. 4G), or "deletion of Chi3l1 significantly reduced mucus layer thickness" (Supplemental Figure 3A-B) are subjective. Since many conclusions were based on imaging data, the authors must provide reliable measures for comparison between conditions, as long as possible, such as fluorescence intensity, area, density, etc, as well as plots and statistical analysis.

Thank you for your insightful suggestions and we have performed the suggested statistical analysis on most of the figures and included the analysis in the revised manuscript (Figure 1A, Figure 3E&F, Supplementary Figure 3B&C). Given large quantity of dietary fiber intertwined with bacteria, it is challenging to make a reliable quantification of bacteria in Figure 4G. However, it is easy to distinguish bacteria from dietary fiber under the

microscope. We have exclusively analyzed gut sections from six mice in each group, and the results are consistent between the two groups.

(2) In the fecal/Lactobacillus transplantation experiments, oral gavage of Lactobacillus to IEC-Chi1 mice ameliorated the colitis phenotype, by preventing colon length reduction, weight loss, and colon inflammation. These findings seem to go against the notion that Chi3l1 is necessary for the colonization of Lactobacillus in the intestinal mucosa. The authors could speculate on how Lactobacillus administration is still beneficial in the absence of Chi3l1. Perhaps, additional data showing the localization of the orally administered bacteria in the gut of Chi3l1 deficient mice would clarify whether Lactobacillus are more successfully colonizing other regions of the gut, but not the mucus layer. Alternatively, later time points of 2% DSS challenge, after Lactobacillus transplantation, would suggest whether the gut colonization by Lactobacillus and therefore the milder colitis phenotype, is sustained for longer periods in the absence of Chi3l1.

Thank you for your thorough review and insightful suggestions. Since we pretreated mice with antibiotics, the intestinal mucus layer is likely damaged according to a previous study (PMID: 37097253). Therefore, gavaged *Lactobacillus* cannot colonize in the mucus layer. Moreover, existing studies have shown that the protective effect of *Lactobacillus* is mainly derived from its metabolites or thallus components, rather than the living bacteria itself (PMID: 36419205, PMID: 27516254).

Zhan M, Liang X, Chen J, Yang X, Han Y, Zhao C, Xiao J, Cao Y, Xiao H, Song M. Dietary 5-demethylnobiletin prevents antibiotic-associated dysbiosis of gut microbiota and damage to the colonic barrier. *Food Funct*. 2023 May 11;14(9):4414-4429. doi: 10.1039/d3fo00516j. PMID: 37097253.

Montgomery TL, Eckstrom K, Lile KH, Caldwell S, Heney ER, Lahue KG, D'Alessandro A, Wargo MJ, Kremmentsov DN. *Lactobacillus reuteri* tryptophan metabolism promotes host susceptibility to CNS autoimmunity. *Microbiome*. 2022 Nov 23;10(1):198. doi: 10.1186/s40168-022-01408-7. PMID: 36419205.

Piermaria J, Bengoechea C, Abraham AG, Guerrero A. Shear and extensional properties of kefir. *Carbohydr Polym*. 2016 Nov 5;152:97-104. doi: 10.1016/j.carbpol.2016.06.067. Epub 2016 Jun 23. PMID: 27516254.

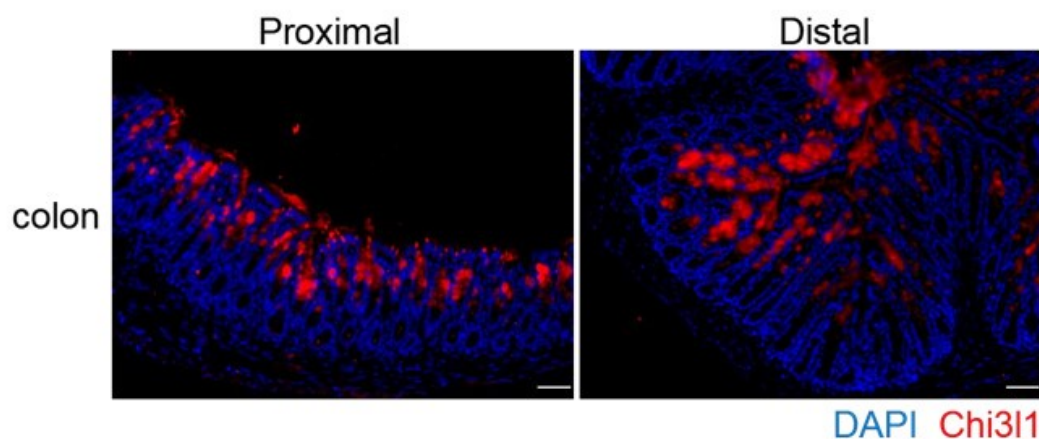
Reviewer #3 (Public Review):

The claim that mucus-associated Ch3l1 controls colonization of beneficial Gram-positive species within the mucus is not conclusive. The study should take into account recent discoveries on the nature of mucus in the colon, namely its mobile fecal association and complex structure based on two distinct mucus barrier layers coming from proximal and distal parts of the colon (PMID:). This impacts the interpretation of how and where Ch3l1 is expressed and gets into the mucus to promote colonization. It also impacts their conclusions because the authors compare fecal vs. tissue mucus, but most of the mucus would be attached to the feces. Of the mucus that was claimed to be isolated from the WT and IEC Ch3l1 KO, this was not biochemically verified. Such verification (e.g. through Western blot) would increase confidence in the data presented. Further, the study relies upon relative microbial profiling, which can mask absolute numbers, making the claim of reduced overall Gram-positive species in mice lacking Ch3l1 unproven. It would be beneficial to show more quantitative approaches (e.g. Quantitative Microbial Profiling, QMP) to provide more definitive conclusions on the impact of Ch3l1 loss on Gram+ microbes.

You raise an excellent point about the data interpretation, and we appreciate your insightful suggestions. We have included the discussion regarding the recent discoveries in the revised manuscript (page 7-8, Line 304-312). According to the recent discovery, the mucus in the proximal colon forms a primary encapsulation barrier around fecal material, while the mucus in the distal colon forms a secondary barrier. Our findings indicate that Chi3l1 is expressed throughout the entire colon, including the proximal, middle, and distal sections (See Author response image 1 below, P.S. Chi3l1 detection in colon presented in the manuscript are from the middle section). This suggests that Chi3l1 likely promotes bacterial colonization across the entire colon. Despite most mucus being expelled with feces, the constant production of mucus and the minimal presence of Chi3l1 in feces (Figure 4C) indicate that Chi3l1 continuously plays a role in promoting the colonization of microbiota.

Author response image 1.

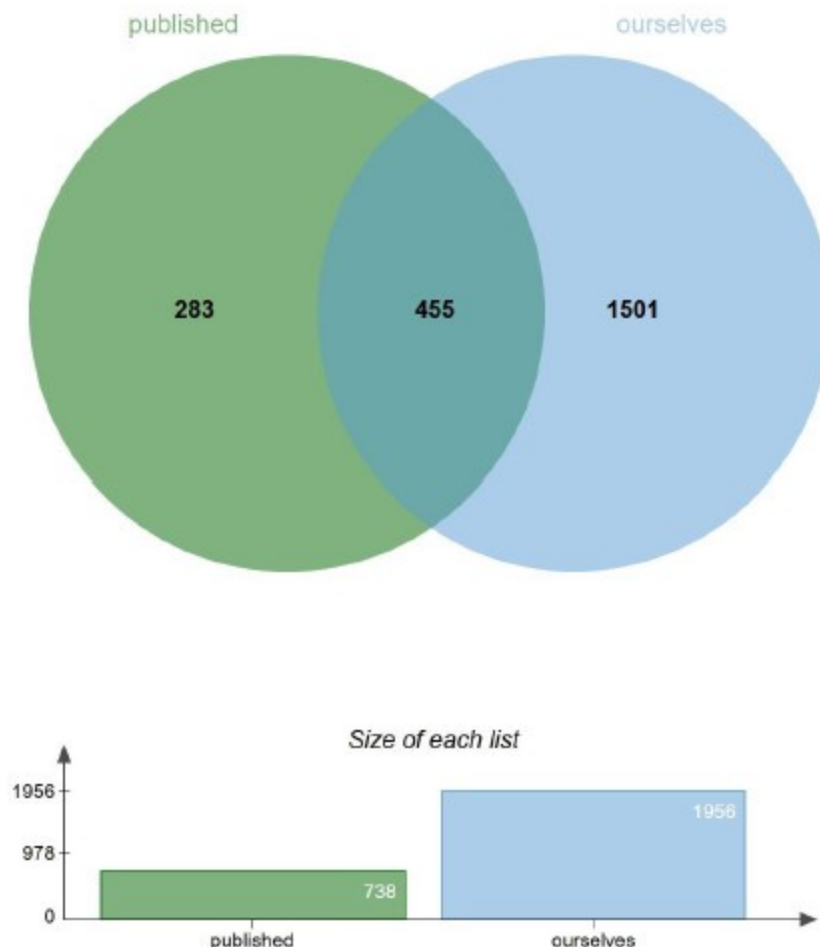
Chi3l1 express in the proximal and distal colon. Immunofluorescence staining on proximal and distal colon sections to detect Chi3l1 (Red) expression. Nuclei were detected with DAPI (blue). Scale bars, 50um.



Given the isolation method of the mucus layer, we followed the paper titled "The Antibacterial Lectin RegIII α Promotes the Spatial Segregation of Microbiota and Host in the Intestine" (PMID: 21998396). Although we did not find a suitable marker representative of the mucus layer for western blotting, we performed protein mass spectrometry on the isolated mucus layers and analyzed the data by comparing it with established research ("Proteomic Analyses of the Two Mucus Layers of the Colon Barrier Reveal That Their Main Component, the Muc2 Mucin, Is Strongly Bound to the Fcgbp Protein," PMID: 19432394). Our data showed a high degree of overlap with the proteins identified in established studies (see Author response image 2 below).

Author response image 2.

Comparison of mucus layer proteins identified by mass spectrometry between Our team and the Hansson team Mucus layer proteins identified by mass spectrometry between our team and the Hansson team (PMID: 19432394) are compared.



Due to a lack of expertise, it has been challenging for us to perform reliable QMP experiments. However, since QMP involves qPCR combined with bacterial sequencing, we conducted 16S rRNA sequencing and confirmed the quantity of certain bacteria by qPCR (revised manuscript, Figure 3B, H, Figure 4E, F, Supplementary Figure 3A). Therefore, our data is reliable to some extent.

Other weaknesses lie in the execution of the aims, leaving many claims incompletely substantiated. For example, much of the imaging data is challenging for the reader to interpret due to it being unfocused, too low of magnification, not including the correct control, and not comparing the same regions of tissues among different in vivo study groups. Statistical rigor could be better demonstrated, particularly when making claims based on imaging data. These are often presented as single images without any statistics (i.e. analysis of multiple images and biological replicates). These images include the LTA signal differences, FISH images, Enterococcus colonization, and mucus thickness.

Thank you for your thorough review and insightful suggestions. We have performed the recommended statistical analysis on most of the figures and included the analysis in the revised manuscript (Figure 1A, Figure 3E&F, Supplementary Figure 3B&C). We have also added arrows in Figure 2B to make the figure easier to understand. Additionally, we repeated some key experiments to show the same regions of tissues among different groups. We will upload higher resolution figures during the revision. Thank you again for your constructive feedback.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

It is recommended to change WT to SPF in the figure and text, as no genetic manipulation was involved in Figure 1.

Thank you for your insightful suggestion. We have also made the suggested modifications to the labeling (revised manuscript, Figure 1A).

Reviewer #2 (Recommendations For The Authors):

The manuscript is well-written, but it would benefit from a critical reading to correct some typos and small grammar issues. Histological and IF images would be more informative if they contained arrows and labels guiding the reader's attention to what the authors want to show. More details about the structures shown in the figures should be included in the legends.

Thank you for your thorough review and insightful suggestions. We have revised the manuscript to correct noticeable typos and grammar issues. Arrows have been added to Figure 2A&B to make the figures easier to understand. Additionally, we have included a detailed description of the structural similarities and differences between chitin and peptidoglycan in the figure legend (revised manuscript, page 19, line 730-733).

Minor points:

- *Page 1, line 36: Please correct "mice models" to "mouse models".*

Thank you for your insightful suggestion and we have made the suggested correction in the revised manuscript (page 1, line 41).

- *Page 3, line 110: "by comparing the structure of chitin with that of peptidoglycan (PGN), a component of bacterial cells walls, we observed that they have similar structures (Fig.2A)". Although both structures are shown side-by-side, no similarities are mentioned or highlighted in the text, figure, or legend.*

Thank you for your insightful suggestion and we have included a detailed description of the structural similarities and differences between chitin and peptidoglycan in the figure legend (revised manuscript, page 19, line 730-733).

- *Fig.5C and Fig.5G: y axis brings "weight (%)". I believe the authors mean "weight change (%)".*

We agrees with your suggestion and has corrected the labeling according to your suggestion (revised manuscript, Figure 5C and G)

- *Page 8: Genotyping method is described as a protocol. Please modify it.*

Thank you for your constructive suggestion and we have modified the genotyping method in the revised manuscript (page 8, line 339-349)

- *Please expand on the term "scaffold model" used in the abstract and discussion.*

Thank you for your thorough review. In this model, Chi3l1 acts as a key component of the scaffold. By binding to bacterial cell wall components like peptidoglycan, Chi3l1 helps anchor and organize bacteria within the mucus layer. This interaction facilitates the colonization of beneficial bacteria such as *Lactobacillus*, which are important for gut health. We included more descriptions regarding scaffold model in the revised manuscript (page 6, line 248-250)

• *Discussion session often recapitulates results description, which makes the text repetitive.*

Thank you for your constructive suggestion and we have removed unnecessary results description in the discussion session in the revised manuscript.

Reviewer #3 (Recommendations For The Authors):

Major comments

(1) *Figure 1A. The staining is very faint, and hard to see. The reader cannot be certain those are Chi3l1-positive cells. Higher Mag is needed.*

Thank you for your insightful suggestion and we have included the higher resolution figures in the revised manuscript Figure 1A.

(2) *The mucus is produced largely by the proximal colon, is adherent to the feces, and mobile with the feces (PMID: 33093110). Therefore it is important to determine where the Chi3l1 is being expressed to be released into the lumen. Further Chi3l1 expression studies are needed to be done in both proximal and distal colon.*

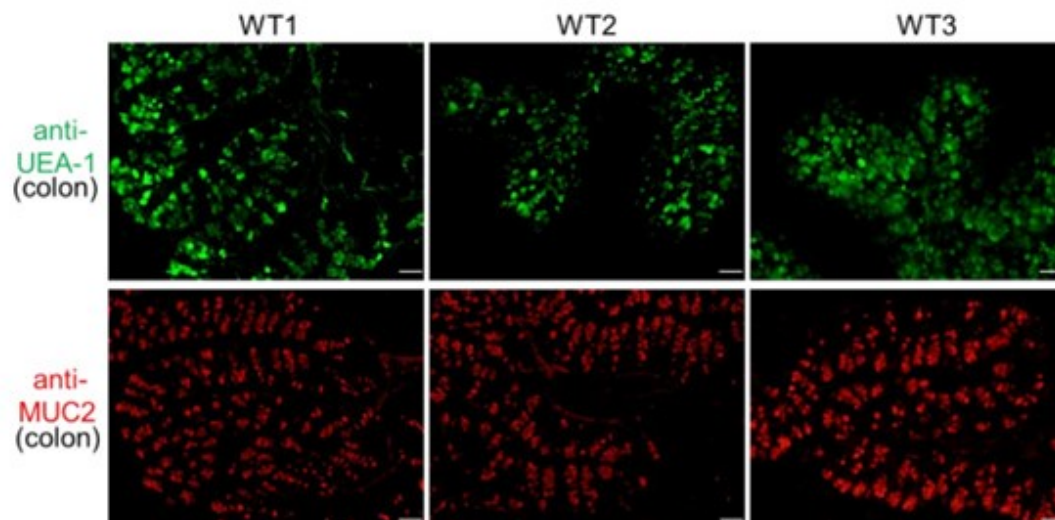
Thank you for your thorough review and insightful suggestions. We have addressed this part in our public review. Additionally, we agree with your suggestions and will conduct further studies on Chi3l1 expression in both the proximal and distal colon.

(3) *Figure 1B. The image is out of focus for the Ileum, and the DAPI signal needs to be brought up for the colon. Which part of the colon is this? The UEA1+ cells do not really look like goblet cells. A better image with clearer goblet cells is needed.*

Thank you for your constructive suggestions. In the revised manuscript, we have included higher-resolution images (Figure 1B). The middle colon (approximately 3 to 4 cm distal from the cecum) was harvested for staining. In addition to UEA-1, we utilized anti-MUC2 antibody to label goblet cells in this colon segment (see Author response image 3 below). The patterns of goblet cells identified by UEA-1 or MUC2 antibodies are similar. The UEA-1-positive cells shown in Figure 1B are presumed to be goblet cells.

Author response image 3.

Goblet Cell Distribution in the Middle Colon. Goblet cells in the middle segment of the colon (approximately 3 to 4 cm distal from the cecum) were detected using immunofluorescence with antibodies against UEA-1 (green) and MUC2 (red). Scale bar=50µm. Representative images are shown from three mice individually stained for each antibody.



(4) Figure 1G. There needs to be some counterstain or contrast imaging to show evidence that cells are present in the untreated sample.

Thank you for your insightful suggestions. We have annotated the cells present in the untreated sample based on the overexposure in the revised manuscript (Figure 1G).

(5) Figure 3B. Is this absolute quantification? How were the data normalized to allow comparison of microbial loads?

Thank you for your thorough review. Figure 3B presents absolute quantification data based on the methodology described in the paper titled "The Antibacterial Lectin RegIII γ Promotes the Spatial Segregation of Microbiota and Host in the Intestine" (PMID: 21998396). Briefly, we amplified a short segment (179 bp) of the 16S rRNA gene using conserved 16S rRNA-specific primers and OP50 (a strain of *E. coli*) as the template. After gel extraction and concentration measurement, the PCR products were diluted to gradient concentrations (0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, 20.48 pg/ μ l). These gradient concentrations were used as templates for qPCR to generate a standard curve based on Ct values and bacterial concentration. The standard curve is used to calculate bacterial concentration in the samples. The data presented in Figure 3B represent the weight of bacteria/milligram sample, calculated as (bacterial concentration x bacterial volume) / (weight of feces or gut content).

(6) Figure 3D. The major case is made for a dramatic reduction in Gram⁺ species, but Figure 1D does not show a dramatic change. Is this difference significant?

Thank you for your thorough review. We don't think we are clear about your question. However, there was no significant difference in Figure 3D. The dramatic reduction in Gram⁺ species are made based on the LTA, Firmicutes FISH, individual species comparison between WT and KO mice, bacterial QPCR results together (Figure 3E-H).

(7) Figures 3E and 3F. These stainings are alone not convincing of reduced Gram⁺ in the KOs. Some stats are required for these images. An independent complementary method is also needed to quantify these with statistics since this data is so central to the study's conclusions.

Thank you for your constructive suggestions. We have included statistical analysis in the revised manuscript (Figure 3E and F). Given large quantity of dietary fiber intertwined with

bacteria, it is challenging to make a reliable quantification of bacteria in Figure 3E. However, it is easy to distinguish bacteria from dietary fiber under the microscope. We have exclusively analyzed gut sections from six mice in each group, and the results are consistent with the Firmicutes FISH results. Complementary method such as bacterial QPCR have been employed to quantify these (Figure 4E, F). Due to a lack of expertise, it has been challenging for us to perform reliable QMP experiments.

(8) Figure 3G. To make quantitative conclusions, the authors need to do quantitative microbial profiling (QMP) of the microbiota. Relative abundance masks absolute numbers, which could be increased. There are qPCR-based QMP platforms the authors could use (PMID: PMIDs: 31940382, 33763385).

Thank you for your constructive suggestions. Due to a lack of expertise, it has been challenging for us to perform reliable QMP experiments. However, since QMP involves qPCR combined with bacterial sequencing, we conducted 16S rRNA sequencing and confirmed the quantity of certain bacteria by qPCR (revised manuscript, Figure 3B, H, Figure 4E, F, Supplementary Figure 3A). In addition to the original bacterial qPCR data presented in the manuscript, we included another bacterial species, *Turicibacter*. Consistent with the 16S rRNA sequencing analysis data, qPCR results showed that *Turicibacter* was more abundant in IECΔChil1 mice than Villin-cre mice (revised manuscript, supplementary Figure 3A, page 4, line 171-173) Therefore, our data is reliable to some extent.

(9) Figure 4B. The data nicely shows Ch3l1 in mucus. However, no data supports the authors' main claim Ch3h1 binds Gram-positive bacteria *in situ*. Dual staining of Ch3l1 with Firmicutes probe would be supportive to show this interaction is happening *in vivo*.

You raise an excellent point, and we agree with your suggestion that we should confirm Chi3l1 binding to Gram-positive bacteria *in situ*. During the study, we attempted dual staining of Chi3l1 with a universal bacterial 16S FISH probe several times, but we were unsuccessful. Despite various optimizations of the protocol, we were only able to detect bacteria, not Chi3l1. It appears that the antibody is not suitable for this method.

(10) Figures 4D - F. Because mucus is associated with feces (PMID:), the data with feces likely contains both Muc2/mucus and Feces. Therefore, it is unclear what the "mucus" is referring to in these figures. To support the authors' conclusions, there needs to be some validation that mucus was purified in the assays. This must be confirmed at a minimum by PAS staining on SDS PAGE gel (should be very high molecular weight) or Western blot with UEA lectin.

Thank you for your insightful suggestions. As mentioned in the public review, the mucus layer was isolated following the protocol described in the paper titled "The Antibacterial Lectin RegIIIy Promotes the Spatial Segregation of Microbiota and Host in the Intestine" (PMID: 21998396). Briefly, after harvesting the middle colon from the mice, we cut open the colon longitudinally. After removing the gut contents, the lumen was vigorously rinsed in PBS while holding one end with forceps. The pellet obtained after centrifuging the rinsate was used as our mucus sample. Fresh feces were collected immediately after the mice defecated in a new, empty cage. We performed Western blot analysis to detect UEA lectin but were unsuccessful.

However, as noted in the public review, we conducted protein mass spectrometry on the isolated mucus layers and analyzed the data by comparing it with established research ("Proteomic Analyses of the Two Mucus Layers of the Colon Barrier Reveal That Their Main Component, the Muc2 Mucin, Is Strongly Bound to the Fcgbp Protein," PMID: 19432394). Our data showed a high degree of overlap with the proteins identified in these established studies.

(11) Figure 4E/F: The units of measurement are in pg/cm², implying picogram per area. Can the authors please explain what this unit is referring to?

We are grateful for your thorough review. The unit pg/cm² represents picograms per square centimeter. Figures 4E and 4F present absolute quantification data based on the methodology described in the paper titled "The Antibacterial Lectin RegIII γ Promotes the Spatial Segregation of Microbiota and Host in the Intestine" (PMID: 21998396). Briefly, we harvested a 3x0.5 cm section of colon and a 9x0.4 cm section of ileum. And then we collected the mucus layer as previously described (responses to question 10). We measured bacterial concentration as described in response to question 5 using the equation ($y = -1.53\ln(x) + 13.581$), where x represents the bacterial concentration and y represents the Ct value. After obtaining the bacterial concentration, we multiplied it by the volume of the rinsate and divided it by the area to obtain the values for pg/cm² used in the figures.

(12) Figure 5E. Normal tissues appear to be from different colon regions from colitis tissues: the "Normal" looks like the proximal colon, while "Colitis" looks like the Distal colon. They cannot be directly compared.

Thank you for your insightful suggestion. We have now included the updated image in the revised manuscript as Figure 5E to compare the same region of the colons.

(13) Similarly, in Figure 5I it appears different colon regions are being compared between groups: Proximal colon in the bottom panels, and distal in the top panels. Since the proximal colon is less damaged by DSS, this data could be misleading.

Thank you for your insightful suggestion. We have now included the updated image in the revised manuscript as Figure 5I to compare the same region of the colons.

(14) In the DSS studies, are the VillinCre and IEC Chit3l1 mice co-housed littermates?

Thank you for your insightful suggestion. In the DSS studies, the Villin-Cre and IEC Δ Chil1 mice are not co-housed littermates. However, they are derived from the same lineage and are housed in the same rack within the same room of the animal facility.

(15) Supplementary Figure 3: Mucus thickness images; are they representative? Stats are needed on multiple mice to support the claim that the mucus is thinner.

Thank you for your insightful suggestion. The images are representative of 4 mice each group. We have now included the statistical analysis in the revised manuscript Supplementary Figure 3C&D.

Minor

(1) Introduction: Reference to "mucosal layer": "Mucosal" and "Mucus" are different things. "Mucosal" refers to the epithelium, lamina propria, and muscularis mucosa. "Mucus" refers to the secreted mucus gel, the focus of the authors' study. Therefore, the statement "mucosal layer" is not proper. "Mucosal layer" should be changed to "mucus layer."

Thank you for your constructive suggestions and we have learned a lot from it. We have made the replacement of "mucosal layer" to "mucus layer" in the revised manuscript.

| (2) Line 366 and related lines: *Feces cannot be "dissolved". "Resuspended" is a better term.*

Thank you for your constructive suggestion and we have made the changes of “dissolved” to “resuspended” in the revised manuscript.

| (3) *Lines 36-37 and 43-44 are redundant to each other.*

Thank you for your constructive suggestion and we have removed the lines 36-37 in the revised manuscript.

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