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# *Bifidobacterium animalis* Causes Extensive Duodenitis and Mild Colonic Inflammation in Monoassociated Interleukin-10 Deficient Mice

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## Abstract

**Background—**We recently showed that *Bifidobacterium animalis* is more prevalent within the colons of IL-10 deficient (−/−) mice than in wild type (WT) animals colonized with the same specific pathogen free (SPF) fecal contents. Here we tested the ability of this organism to cause T cell-mediated intestinal inflammation by introducing it into germ-free (GF) IL-10−/− mice.

**Methods—**GF IL-10−/− or WT mice were monoassociated with *Bifidobacterium animalis* subsp. *animalis* ATCC 25527$^T$ or with *Bifidobacterium infantis* ATCC 15697$^T$. Inflammation was measured by blinded histologic scores of the duodenum, cecum and colon and by spontaneous secretion of IL-12/IL-23 p40 from colonic explants. Bacterial antigen-specific CD4$^+$ mesenteric lymph node (MLN) T cell recall responses were measured in response to antigen presenting cells (APC) pulsed with bacterial lysates.

**Results—***B. animalis* caused marked duodenal inflammation and mild colitis in monoassociated IL-10−/− mice, whereas the intestinal tracts of WT animals remained free of inflammation. *B. infantis* colonization resulted in mild inflammation in the duodena of IL-10−/− mice. CD4$^+$ MLN T cells from *B. animalis* monoassociated IL-10−/− mice secreted high levels of IFN-γ and IL-17 in response to *B. animalis* lysate. *B. animalis* equally colonized the different intestinal regions of WT and IL-10−/− mice.

**Conclusions—***B. animalis*, a traditional probiotic species that is expanded in experimental colitis in this model, induces marked duodenal and mild colonic inflammation and TH1/TH17 immune responses when introduced alone into GF IL-10−/− mice. This suggests a potential pathogenic role for this commensal bacterial species in a susceptible host.

### Keywords

Intestinal inflammation; animal models; IL-10 deficient mice; *Bifidobacterium animalis*

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## INTRODUCTION

Human inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are believed to be caused by an inappropriate cell-mediated (T cell) immune response to commensal enteric bacteria in genetically susceptible individuals [1–2] This is supported by clinical observations linking increased concentrations of luminal and adherent bacteria to inflamed regions of the intestinal tract [3–5] and by studies of experimental colitis in genetically susceptible animals, including IL-10 deficient (−/−) mice, where colitis and immune activation fail to develop in the absence of commensal bacteria [1, 6]. Colonization of susceptible gnotobiotic rodents has demonstrated that some commensal bacterial species cause inflammation, some have no effect, and others (probiotics) provide protection from the inflammation caused by detrimental species [3]. For example, gnotobiotic IL-10−/− mice develop bacterial species-antigen specific T cell-mediated intestinal inflammation when monoassociated with *Enterococcus faecalis* or *Escherichia coli* [7], but remain healthy when colonized with *Bacteroides vulgatus*, which has been shown to induce colitis in HLA-B27/β2 microglobulin transgenic rats [8]. IL-10−/− mice housed under specific pathogen free (SPF) conditions and fed *Lactobacillus salivarius* subsp. *salivarius* or *Bifidobacterium infantis* displayed reduced colonic inflammation and attenuated Peyer's patch IFN-γ responses following *in vitro* stimulation with the enteric pathogen *Salmonella typhimurium*, compared to placebo controls [9]. These observations highlight the importance of understanding how different intestinal inhabitants selectively impact the host's mucosal immune system, and how this immune activation or inhibition influences health in hosts with distinct genetic backgrounds.

We recently reported significant alterations in the composition of the enteric microbiota in colitic IL-10−/− mice compared to healthy WT animals after germ-free (GF) mice were colonized with fecal microbiota obtained from SPF wild type (WT) mice [10]. Changes in microbiota composition with progression of colitis were analyzed using PCR and denaturing gradient gel electrophoresis (PCR/DGGE) and specific DNA:RNA dot blot analysis. Interestingly, *Bifidobacterium animalis* was one of four bacterial species that increased in abundance as colitis developed. Bifidobacteria are widely considered to be beneficial commensal organisms that are "generally regarded as safe" (GRAS) and have been associated with probiotic activity [3, 11–13]. Therefore, the increased prevalence of *B. animalis* in the colons of colitic IL-10−/− mice was unexpected and the inflammatory potential of this species in IL-10−/− mice warranted further investigation.

In the current study, we evaluated *B. animalis* for its ability to induce intestinal inflammation in gnotobiotic WT and IL-10−/− mice using the type strain *Bifidobacterium animalis* subsp. *animalis* ATCC 25527$^T$ for colonization. For comparison, we also colonized gnotobiotic WT and IL-10−/− mice with *Bifidobacterium infantis* ATCC 15697$^T$. We further investigated the antigen specificity of the T cell-mediated immune response of *B. animalis* colonized mice and assessed possible cross-reactivity of the T cell response to other bacteria. Here we report that IL-10−/− mice monoassociated with *B. animalis* developed extensive duodenal and mild colitic inflammation with CD4$^+$ T cell responses directed against both unique and shared bacterial antigens expressed by *B. animalis*.

## MATERIALS AND METHODS

### Bacterial strains and media

*Bifidobacterium animalis* subsp. *animalis* ATCC 25527$^T$, *Bifidobacterium infantis* ATCC 15697$^T$ and *Bifidobacterium bifidum* ATCC 11863$^T$ were obtained from the American Type Culture Collection (ATCC) and propagated anaerobically at 37°C in Lactobacilli MRS medium (BD Biosciences, Sparks, MD). A murine *Escherichia coli* strain designated

NC1017 and a human oral isolate of *Enterococcus faecalis* (strain OG1RF provided by Mark Huycke M.D.) 14 have been previously described. Bacterial lysates were prepared as previously described 7.

## Mice

GF IL-10−/− mice on the 129S6/SvEv background and WT control (inbred 129S6/SvEv) mice were from breeding colonies maintained at the National Gnotobiotic Rodent Resource Center (NGRRC), University of North Carolina, Chapel Hill or the Center for Gastrointestinal Biology and Disease (CGIBD) Gnotobiotic Animal Core at North Carolina State University. These mouse colonies were originally derived by hysterectomy at the Gnotobiotic Laboratory (University of Wisconsin, Madison) 6. Mice were monoassociated at 10–26 weeks of age with *B. animalis* or *B. infantis* by gavage feeding and rectal swabbing with viable cultured bacteria. Monoassociated mice were maintained in either the NGRRC or the CGIBD gnotobiotic facilities. Mice colonized with *B. animalis* or *B. infantis* were housed in separate isolators. Bacterial monoassociation and absence of contamination by other bacterial species were confirmed by periodic aerobic and anaerobic culture of stool samples. Mice were killed 11, 17 or 20–24 weeks after colonization with *B. animalis* and 22–23 weeks after colonization with *B. infantis*. WT 129S6/SvEv mice (Taconic Laboratories, Germantown, NY) maintained under SPF conditions free of *Helicobacter* species were used to prepare antigen-presenting cells (APC). Animal use protocols were approved by the Institutional Animal Care and Use Committees of North Carolina State University and the University of North Carolina at Chapel Hill.

## Investigation of bacterial colonization of mice

At necropsy after 24 weeks of colonization with *B. animalis*, a portion of the cecal contents from two 129 WT and two IL-10−/− mice was used to inoculate sterile Lactobacilli MRS liquid media and cultures were incubated in sealed glass culture tubes overnight at 37°C. Fecal samples were collected from two 129 WT and two IL-10−/− *B. infantis* monoassociated mice after 8 weeks of colonization and were similarly inoculated into sterile Lactobacilli MRS liquid media. An aliquot from each culture was streaked onto Lactobacilli MRS agar supplemented with 0.05% L-cysteine and plates were incubated for 72 hrs under anaerobic conditions. Cell and colony morphology of these isolates were identical to the *B. animalis* and *B. infantis* strains initially used to colonize the animals (data not shown). Genomic DNA was isolated from randomly selected individual colonies of *B. animalis* and *B. infantis* stock cultures and from each *ex vivo* isolate using the DNeasy Blood & Tissue Kit (Quiagen, Valencia, CA). 16S ribosomal RNA gene sequence was amplified by PCR using the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1491R (GGTTACCTTGTTACGACTT). The following reagents were included in each PCR tube: 5 μL 10 × buffer, 1.5 μL 50 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.5 μL 5 mM dNTP (GE Healthcare, Piscataway, NJ), 1 μL each of primers 27F and 1491R (5 pmol/μL), 30–40 ng DNA template, 1.25 U Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and deionized water to a final volume of 50 μL. The PCR cycling was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA), under the following conditions: 95°C for 2 min and 30 sec, then 35 cycles consisting of 95°C for 60 s, 51°C for 60 s and 72°C for 120 s. The PCR products were incubated at 4°C until used. Sequencing of PCR products was carried out using 27F and 1491R as sequencing primers. DNA was sequenced at the UNC-CH Genome Analysis Facility on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) confirmed that all *ex vivo* isolates from mice monoassociated with *B. animalis* or *B. infantis* were *B. animalis* and *B. infantis*, respectively.

Bacterial numbers of *B. animalis* in the gut of 129S6/SvEv WT and IL-10−/− mice (six mice each) were measured in the duodenum, cecum, proximal colon and distal colon by quantitative plating of serial dilutions of homogenized intestinal segments on Lactobacilli MRS agar supplemented with 0.05% L-cysteine (Sigma, St. Lois, MO). Plates were incubated for 72 hrs under anaerobic conditions at 37°C and colonies were enumerated.

### Histological scoring

Sections of colon (proximal and distal), cecum and duodenum were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin and stained with H&E. Sections were scored blindly for severity of inflammation by a single individual using a well-validated scale 6, 7, 15, 16. Histological scores (0 to 4) were based on the degree of lamina propria and submucosal mononuclear cellular infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion. These scoring criteria that were developed previously to evaluate colitis were modified for use with the duodenal tissue sections. In the duodenum a score of 0 represents no inflammation and normal villus architecture. A score of 1 represents mild focal cellular infiltration and normal villous architecture. A score of 2 represents mild lamina propria cellular infiltration and early crypt epithelial hyperplasia with normal villus architecture. A score of 3 represents more pronounced cellular infiltration, thickened mucosa, marked epithelial hyperplasia and moderate distortion of villus architecture. A score of 4 represents extensive cellular infiltration throughout the section and severe architectural distortion.

### Colonic tissue fragment cultures

Colonic tissue fragment cultures were prepared from the large intestine as previously described 6, 7. Colonic tissue was thoroughly irrigated with phosphate-buffered saline (PBS), shaken at room temperature in RPMI containing 50 μg/mL gentamicin for 30 minutes at 280 rpm, cut into 1-cm fragments, blotted to remove excess media, and weighed. Colonic tissue fragments were distributed (0.05 g per well) into 24-well plates (Costar 3524) and incubated in 1 mL of RPMI 1640 medium supplemented with 5% fetal bovine serum, 50 μg/mL gentamicin, and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B; Invitrogen, Carlsbad, CA) for 20 hours at 37°C. Supernatants were collected and stored at −20°C prior to use for IL-12/IL-23 p40 quantification by ELISA.

### $CD4^+$ T cell isolation

$CD4^+$ T cells were enriched by negative selection from mesenteric lymph node (MLN) cells harvested from IL-10−/− or WT mice using magnetic beads coated with B cell specific (anti-CD45R) antibody plus beads coated with CD8-specific (anti-CD8α) antibody according to the manufacturer's instructions (Miltenyi - Biotec, Auburn, CA) as previously described 7, 16. Over 95% of the enriched MLN cell populations expressed CD4 as determined by flow cytometry.

### Antigen-presenting cell preparation and $CD4^+$ T cell stimulation

Splenic antigen presenting cells (APC) were prepared from SPF 129S6/SvEv WT mice and pulsed overnight with 10 μg/mL of *B. animalis*, *B. bifidum*, *B. infantis*, *Enterococcus faecalis*, or *Escherichia coli* lysate or keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) as an unrelated antigen control, as described previously7, 16. $CD4^+$ T cells ($2 \times 10^5$ $CD4^+$ T cells/well) were co-cultured for 72 hours with antigen-pulsed APC ($3 \times 10^5$ APC cell/well) in flat bottom 96-well cell culture plates (Costar 3595), 0.2 mL per culture. Supernatants were collected after 3 days and stored at −20°C.

### Dendritic cell stimulation with bacterial cell lysates

Bone marrow derived dendritic cells (BMDC) were isolated and cultured from femora and tibiae of SPF 129S6/SvEv mice, as previously described 17. BMDC ($2 \times 10^4$) were seeded in triplicate wells of flat bottom 96-well cell culture plates (Costar 3595) in the presence of media alone, LPS (1 μg/ml) or various bacterial lysates (10 μg/ml) at 0.2 mL per culture. Supernatants were collected after 3 days and stored at −20°C.

### Cytokine measurements

We used commercially available monoclonal anti-mouse IFN-γ, IL-12/IL-23 p40 (BD Biosciences Pharmingen, San Diego, CA), and IL-17 (e-Bioscience, San Diego, CA) specific capture and detection reagents to measure amounts of secreted cytokines by ELISA. For detection of IL-12/IL-23 p40, plates were coated with anti-mouse IL-12 p40/p70 (clone C15.6) and bound ligand was detected with biotin anti-mouse IL-12 p40/p70 (clone C17.8). To quantify IFN-γ, plates were coated with anti-mouse IFN-γ (clone R4-6A2) and bound ligand was detected with biotin anti-mouse IFN-γ (clone XMG1.2). For detection of IL-17, plates were coated with anti-mouse IL-17A, clone eBioTC11-18H10.1 and IL-17 was detected using biotin anti-mouse IL-17A, clone eBioTC11-8H4. Cytokine levels were measured in triplicate supernatants and compared to standard curves generated using recombinant murine cytokines.

### Flow Cytometry

MLN cell subpopulations (before and after negative selection) were evaluated as previously described 7 on the FACScan (BD Biosciences, Mountain View, CA) using FITC anti-CD4, PE anti-CD8, or FITC anti-B220 (all from Invitrogen).

### Statistical analyses

Nonparametric histologic scores were analyzed with the Mann Whitney test (SAS; Cary, NC) by statisticians in the Biostatistics Core of the Center for Gastrointestinal Biology and Disease. The paired Student's T-test was used to compare all other data. Statistical significance was defined as $p < 0.05$ for comparisons indicated. Data are reported as mean values ± standard error of the mean (SE), unless otherwise indicated.

## RESULTS

### *B. animalis* monoassociated IL-10−/− mice develop mild colitis and marked inflammation in the duodenum

To test whether *B. animalis* could elicit intestinal inflammation in IL-10−/− mice, we colonized inbred GF IL-10−/− (129S6/SvEv background) and GF 129S6/SvEv WT mice with *B. animalis* ATCC $25527^T$ for 20–24 weeks. We confirmed monoassociation by analyzing the 16S ribosomal RNA sequence of several *ex vivo* bacterial isolates from cecal contents at necropsy (data not shown). Two of nineteen monoassociated IL-10−/− mice studied developed rectal prolapse, however most of the mice did not display outward signs of distress beyond lethargy and soft stools. Histological evidence of mild inflammation was apparent throughout the colons of IL-10−/− mice, characterized by moderate crypt hyperplasia, infiltration of predominantly mononuclear cells into the lamina propria, but not the submucosa, and moderate goblet cell depletion (Figure 1D, E, and F). WT 129S6/SvEv mice, similarly colonized for 20–24 weeks had no histological evidence of colitis (Figure 1A, B, and C). Importantly, there was considerable grossly evident thickening of the duodena in IL-10−/− mice with evidence of partial obstruction, demonstrated by gastric and proximal duodenal distention with fluid and luminal content retention (Figure 2B ). However, there were no significant differences in the weights of age matched WT and

IL-10−/− mice at 20–24 weeks after colonization, suggesting the absence of high grade duodenal obstruction (data not shown). Histological examination of duodenal tissue revealed massive cellular infiltration of mononuclear cells into the lamina propria, including the villi and significant crypt hyperplasia, compared to healthy duodenal tissue from monoassociated WT animals (compare IL-10−/−, Figure 2D–E with WT Figure 2C). The typical architecture of the mucosa was altered by the formation of abnormal crypt and villus structures consisting of branched and fused villi, but no mucosal ulceration (Figure 2D–E). This inflammation was localized to the duodenum and did not extend into the jejunum or ileum (data not shown). Histological scores for the colons of IL-10−/− mice reflected a mild pancolitis in these animals (distal colon: 1.9 ± 0.9; proximal colon: 1.6 ± 0.8; cecum: 2.3 ± 0.6) and were significantly higher than the scores for WT colons (distal colon: 0.7 ± 0.2; proximal colon: 1.0 ± 0.5; cecum: 1.2 ± 0.4) (Figure 3A). This histologic evidence of colitis in *B. animalis*-monoassociated IL-10−/− mice was further supported by the spontaneous release of higher amounts of IL-12/IL-23 p40 from colonic explants from IL-10−/− mice compared to WT mice (Figure 3B). Scores for histologic detection of inflammation in the duodenum reflected the extensive abnormalities within this intestinal segment in IL-10−/− animals (3.1 ± 0.3) compared to WT duodenal tissue (0.6 ± 0.2) (Figure 3A). In preliminary experiments we evaluated WT and IL-10−/− mice monoassociated with *B. animalis* for 11 and 17 weeks. Low levels of inflammation were detected in the distal colons and ceca of IL-10−/− animals by histological analysis and by spontaneous IL-12/IL-23 p40 release from colonic explants (data not shown). Duodena were not evaluated at these earlier time points. Together, these results show that IL-10−/− mice monoassociated with *B. animalis* developed a mild pancolitis and extensive duodenal inflammation with proximal duodenal and gastric dilation, whereas similarly colonized WT mice remained healthy.

### *B. infantis* monoassociated IL-10−/− mice develop low-grade colonic and duodenal inflammation

We wondered whether *B. animalis* was unique among bifidobacteria in its ability to induce intestinal inflammation in IL-10−/− mice, or whether other *Bifidobacterium* species shared this capacity. Therefore, we colonized GF WT and IL-10−/− mice with *B. infantis* for 22–23 weeks. All WT and IL-10−/− mice appeared healthy throughout the colonization period and lacked macroscopic evidence of intestinal inflammation or gastric or duodenal dilation. Histological scores for different regions of the intestinal tract and spontaneous IL-12/IL-23 p40 secretion from colonic explants reflected a low level inflammatory response in IL-10−/− animals (Table 1). Only the duodenal histologic scores showed significant differences between IL-10−/− and WT mice, with no differences in the colon. Importantly, these measurements of inflammation in the intestines of *B. infantis* monoassociated IL-10−/− animals were lower than those obtained from *B. animalis* monoassociated IL-10−/− animals shown in Figure 3.

### The distribution of inflammation among intestinal compartments in *B. animalis* monoassociated IL-10−/− mice is not explained by bacterial colonization patterns

We hypothesized that the severe inflammation seen within the duodena of IL-10−/− mice might be due to selective colonization of this compartment by *B. animalis*. To test this we performed quantitative bacterial cultures on homogenized intestinal segments from the distal colon, proximal colon, cecum and duodenum of WT and IL-10−/− mice. This technique measures both luminal and adherent bacteria colonizing each compartment 18. Comparing WT and IL-10−/− mice, there were no statistically significant differences in the numbers of viable bacteria colonizing any of these compartments (Table 2). Also, bacterial concentrations were 2–3 orders of magnitude higher in the colons than in the duodena of these animals. These data suggest that the severe inflammation observed within the duodena

of IL-10−/− mice compared to healthy WT mice was not due to differential colonization of this compartment by *B. animalis*.

### CD4$^+$ MLN T cell response to *B. animalis* displays bacterial species-antigen specificity

We have previously shown that IL-10−/− mice monoassociated or dual-associated with bacterial species that cause intestinal inflammation display CD4$^+$ MLN T cell responses to lysates of the species that induces inflammation with bacterial species-antigen specificity 7, 19. Here we asked whether *B. animalis* monoassociated IL-10−/− mice developed similar T cell responses directed against *B. animalis* bacterial antigens and whether T cells would cross-react with antigens expressed by other bacterial species. MLN-derived CD4$^+$ T cells (isolated from IL-10−/− and WT monoassociated mice) were cultured with WT APC that had been pulsed *in vitro* with either KLH (as an unrelated antigen control) or with *B. animalis, B. infantis, B. bifidum, E. coli* or *E. faecalis* lysates. CD4$^+$ MLN T-cells from IL-10−/− mice, but not from WT mice, produced high levels of IFN- γ (Figure 4A) and IL-17 (Figure 4B) when stimulated with *B. animalis* lysate-pulsed APC, indicating a strong recall T cell response against bacterial antigens expressed by this organism. Interestingly, these IL-10−/− T cells also secreted moderate levels of IFN-γ and IL-17 in response to APC pulsed with lysates of *B. infantis, B. bifidum* and *E. coli*, but not to *E. faecalis* lysate-pulsed APC. Cytokine measurements from CD4$^+$ T cells from monoassociated WT mice cultured with the same lysates were consistently at or below the detection limits for these assays.

Bacterial cell lysates contain many components that could influence T cell secretion of inflammatory cytokines through their interaction with pattern recognition receptors on APC in an antigen non-specific manner 20. In order to evaluate the adjuvant-mediated stimulating capacity of these lysates we cultured WT BMDC in the presence of media alone, LPS (1 μg/ml) or each bacterial lysate at the same concentration used to pulse APC prior to co-culture with CD4$^+$ T cells (10 μg/ml) and measured the secretion of IL-12/IL-23 p40 (Table 3). LPS and *E. coli* lysate stimulated the highest levels of IL-12/IL-23 p40 from BMDC (13,307.7 ± 1,975.9 and 15,307.7 ± 4,137.4 pg/ml, respectively); the three *Bifidobacterium* lysates stimulated approximately 15 fold lower levels and *E. faecalis* stimulated only slightly more than media alone. These results indicate that the three different *Bifidobacterium* lysates share similar potential for adjuvant activity. Therefore, IL-10−/− mice monoassociated with *B. animalis* likely develop CD4$^+$ T cell immune responses against antigens expressed by *B. animalis* that are either not shared or are expressed at lower levels by the other species of bifidobacteria that we evaluated (Figure 4).

## DISCUSSION

Our study demonstrates that *Bifidobacterium animalis* subsp. *animalis* ATCC 25527$^T$ causes mild colitis and marked duodenal inflammation in monoassociated gnotobiotic IL-10−/− mice. These results provide potential pathogenetic significance for our earlier study that showed increased abundance of *B. animalis* in the colons of SPF IL-10−/− mice as cecal and colonic inflammation progressed 10. We demonstrate that a bacterial species that is selectively expanded in SPF IL-10−/− mice with experimental colitis 10 is capable of inducing colitis and TH1 and TH17 CD4$^+$ T cell responses in monoassociated gnotobiotic IL-10−/− mice.

*B. animalis* now joins *E. faecalis*, 7, 14 *E. coli* (mouse isolate), 7 and *Enterobacter cloacae* 21 in a growing list of commensal organisms shown to initiate intestinal inflammation in IL-10−/− mice. Importantly, many commensal isolates are not able to cause intestinal inflammation in monoassociated IL-10−/− mice, including: *Pseudomonas fluorescens* 7, *Bacteroides vulgatus* (guinea pig), *Streptococcus faecium* [Group D], *Escherichia coli*, *Peptostreptococcus productus*, *Eubacterium contortum*, and *Streptococcus avium* (isolates

from Crohn's patients)6, *Helicobacter hepaticus*22, *Candida albicans*, *Lactococcus lactis*, a *Bifidobacterium* species, a *Bacillus* species, several species of *Lactobacillus*,14 *Viridans* group *streptococci*, and *Clostridium sordellii* 23. In our study, GF IL-10−/− mice monoassociated with *B. infantis* developed mild inflammation in the duodenum after 22–23 weeks, but there were no statistically significant differences in the histological scores from the ceca and colons of WT *B. infantis*-colonized animals. The studies available to date demonstrate considerable heterogeneity within bacterial genera and even within individual species in the capacity to induce intestinal inflammation in GF IL-10−/− mice. For example, a commensal strain of *E. coli* (designated NC 101) isolated from a SPF-housed 129S6/SvEv WT mouse caused moderate colitis in GF IL-10−/− mice 7, whereas an *E. coli* strain isolated from a patient with Crohn's disease 6 and a laboratory strain, E. coli K12 24 did not. Similar diversity appears to exist among various isolates/species of *Bifidobacterium*, since the *B. animalis* ATCC 25527$^T$ strain used in our study induced marked duodenal and mild colonic inflammation in GF IL-10−/− mice, *B. infantis* 15697$^T$ induced a low-grade response and an isolate used by Balish *et. al*. did not cause intestinal inflammation 14. Indeed, significant genetic and phenotypic heterogeneity has been reported for different species and strains within the genus *Bifidobacterium* 25, 26.

Our observation that *B. animalis* causes intestinal inflammation in this genetically susceptible model is of considerable interest, since this species is widely utilized as a probiotic organism. Much of the clinical research has been done using the strain *Bifidobacterium animalis* DN-173 010 12. This strain has a high postgastric survival rate that is greater than another commercially available *Bifidobacterium* strain 27, 28. *B. animalis* DN-173 010 also reduced colonic transit time when consumed in *Bifidobacterium*-fermented milk 29. Van der Meulen *et al*, reported *B. animalis* DN-173 010 to be unique among *B. animalis* isolates in its preferential metabolism of certain short-chain fructans, suggesting it may have a competitive advantage over other microorganisms in the human gut, where oligo- and polysaccharides are the main sugar substrates 30. Recently, *B. animalis* has been reclassified to include the two subspecies *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium animalis* subsp. *lactis* based on molecular and phenotypic characteristics 26. Strains of the *lactis* subspecies including BB12 and HN019 have variable probiotic effects in animals and humans 31–35. Following our previous observation that *B. animalis* forms an increased proportion of the gut microbiota of colitic IL-10−/− mice compared with WT mice 10, we selected strain ATCC 25527$^T$ to colonize GF animals because of its designation as the type strain for *B. animalis*. Our results suggest that the strain ATCC 25527$^T$ possesses unique phenotypic traits that allow it to initiate an inflammatory response under certain circumstances, including monoassociation of IL-10 deficient rodents.

The mechanisms underlying the ability of some commensal bacterial strains to cause inflammation in susceptible hosts, while others remain innocuous are currently unknown. One possibility is that detrimental species colonize genetically susceptible individuals to a greater extent than non-susceptible individuals, thereby providing increased stimulus for the innate and adaptive immune systems 2. However, quantitative bacterial culture from different intestinal segments revealed no difference in *B. animalis* colonization patterns between monoassociated WT and IL-10−/− mice. Furthermore, *B. animalis* colony counts from the duodena of both WT and IL-10−/− mice were 2–3 orders of magnitude lower than those obtained from the colon. We previously reported moderate duodenal inflammation with functional obstruction in IL-10−/− mice monoassociated with *E. faecalis*, but not with *E. coli* NC101 despite higher duodenal concentrations of *E. coli* vs. *E. faecalis*, thus demonstrating a similar disassociation of regional intestinal inflammation and luminal bacterial concentrations 7. The quantitative bacterial culture technique used in the current study measures both luminal and adherent bacteria 18 but does not distinguish the relative

proportion of adherent organisms, which has been reported to correlate with regional intestinal inflammation in colonic biopsies 4.

The extensive inflammation and possible partial obstruction observed in the duodena of *B. animalis*- monoassociated IL-10−/− mice is noteworthy. IL-10−/− mice housed in our SPF facility lack duodenal inflammation 6 although Kuhn *et al* described extensive duodenal and small bowel intestinal inflammation in IL-10−/− mice housed under conventional, but not SPF conditions 36. The moderate duodenitis observed by Kim *et al* in *E. faecalis*-monoassociated IL-10−/− mice was not noted until after 30 weeks of colonization, whereas distal colitis was first observed at 10–12 weeks and was well advanced by 22 weeks 7. In contrast, *B. animalis* colonized IL-10−/− mice exhibit extensive duodenal inflammation after 20–24 weeks with only mild inflammation in the colon. GF HLA-B27 transgenic rats develop mild/moderate antral and proximal duodenal inflammation in addition to severe colitis one month after colonization with SPF fecal microbiota, but only mild/moderate colitis and no duodenal inflammation after monoassociation with *Bacteroides vulgatus* 15. Dohi *et al* described a model of chronic gastroduodenitis in TCR −/− mice housed under SPF conditions that were transfer recipients of IL-4−/− $CD4^+CD45RB^{hi}$ T cells 37. These mice developed gastroduodenitis and colitis without obvious changes in the jejunum or ileum. Interestingly, the gastroduodenitis was only partially blocked with antibiotic treatment, whereas, colitis was completely inhibited, suggesting a role for nonviable antigens in the duodenal compartment 37. This is consistent with the observations that SAMP-1/Yit Fc mice continue to exhibit ileitis 38 and IL-2 deficient mice have gastroduodenitis 39 in GF conditions, although mice of both types have attenuated colonic inflammation in the absence of viable bacteria. The explanation for more active inflammation in the duodenum compared to other intestinal compartments that we evaluated remains unclear. One possibility is that incompletely degraded food components and fecal bacteria ingested through coprophagia could increase exposure of the duodenal mucosa to nonviable *B. animalis* antigens and adjuvants, thereby recruiting pathogenic immune cells to this region. As stated above, it is also possible that there is a difference in the extent of mucosal epithelial adherence of *B. animalis* in the duodenum compared to the colon, which warrants further study.

We demonstrate that $CD4^+$ MLN T cells from *B. animalis* monoassociated IL-10−/− mice, but not WT mice, secrete IFN-γ and IL-17 when restimulated *in vitro* with APC previously incubated with *B. animalis* bacterial lysate. These T cells also secreted the same two cytokines, albeit to a lesser extent, in response to APC pulsed with lysates of *B. infantis*, *B. bifidum* and *E. coli*, but not *E. faecalis* bacterial lysate or an unrelated protein antigen, KLH. Therefore, there appears to be some immunologic cross-reactivity and/or shared bacterial antigens among the three *Bifidobacterium* species and *E. coli*. The higher recall response to *B. animalis* than to *B. infantis* or *B. bifidum* suggests that mucosal $CD4^+$ T cells react to unique antigens expressed by *B. animalis* that are not shared by other *Bifidobacterium* species. Thus, antigenic heterogeneity among different bacterial isolates may influence their potential to initiate an inflammatory response. However, the presence of an immunogenic bacterial antigen may not be sufficient to cause inflammation. Sydora *et al* demonstrated that IL-10−/− mice monoassociated with *Bacteroides vulgatus* did not develop intestinal inflammation, but did generate a strong systemic T cell response to *B. vulgatus* antigens 23. The authors recently went on to show that disruption of the intestinal epithelial barrier of *B. vulgatus* monoassociated IL-10−/− mice with indomethacin allowed the bacteria to initiate and sustain an intense intestinal inflammatory response 40. These studies suggest that in order to cause inflammation, bacteria may require the capacity to breach the epithelial barrier to interact with lamina propria antigen presenting cells such as dendritic cells. Whether *B. animalis* ATCC $25527^T$ is more invasive than other bifidobacteria remains to be determined. We evaluated the adjuvant stimulating capacity of our bacterial lysates and

found no difference in the ability of the three *Bifidobacterium* lysates to stimulate IL-12/IL-23 p40 secretion by BMDC, further supporting our conclusion that the strong T cell recall response to *B. animalis* represents true bacterial antigen specificity.

The inflammation initiated by *Bifidobacterium animalis* subsp. *animalis* ATCC 25527$^T$ is characterized by a mild pancolitis and marked duodenitis. The fact that duodenal inflammation is not observed in IL-10−/− mice housed under SPF conditions 6 suggests that either the *B. animalis* that expand in these animals with disease progression are in some way different from strain ATCC 25527$^T$, that the microbiota in the proximal gut of SPF mice prevent *B. animalis* from colonizing, attaching to or invading the duodenum, or that other members of the microbial community stimulate or inhibit the inflammatory response in a manner that is not reproduced by simple monoassociation. In conventional mice, the duodenum is populated by high numbers of lactobacilli 41 and these organisms can prevent inflammation in various animal models of IBD 11. Therefore, it is possible that lactobacilli prevent *B. animalis* from colonizing, attaching to the epithelium or invading the duodenum in SPF animals or may exert an anti-inflammatory effect at this site to prevent visible signs of duodenitis.

In summary, we have demonstrated that *B. animalis*, which has previously been shown to represent an increased proportion of the gut microbiota of colitic IL-10−/− mice, can induce intestinal inflammation and recall TH1 and TH17 immune responses after these animals have been monoassociated. This suggests that *B. animalis* may contribute to the colitis observed in SPF IL-10−/− mice. We do not suggest that our study implicates *B. animalis* as an unsafe probiotic organism for human consumption. Most strains utilized in this manner have undergone rigorous testing for safety and have no documented adverse effects 13, 42. However, our results indicate that even enteric bacterial species that are traditionally viewed to have probiotic activities also posess the potential to contribute to intestinal inflammation in a susceptible host.

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## Abbreviations

| | |
|---|---|
| **APC** | Antigen presenting cell |
| **GF** | germ-free |
| **IFN-γ** | interferon-gamma |
| **IL** | interleukin |
| **IL-10−/−** | interleukin-10 deficient |
| **MLN** | mesenteric lymph node |

| | |
|---|---|
| **SPF** | specific pathogen free |
| **TG** | transgenic |
| **WT** | wild-type mouse |

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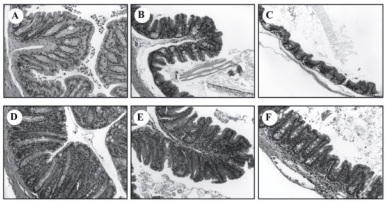


**Figure 1. Histological evidence of inflammation in the colons of *B. animalis* monoassociated mice**

WT (A–C) and IL-10−/− (D–F) mice were monoassociated with *B. animalis* for 23 weeks. At necropsy portions of distal colon (A and D), proximal colon (B and E) and cecum (C and F) were removed. H & E stained paraffin sections reveal mild inflammation in the colons of IL-10−/− mice, characterized by crypt hyperplasia, goblet cell depletion, and lamina propria cellular infiltration. All images are at 20X magnification.

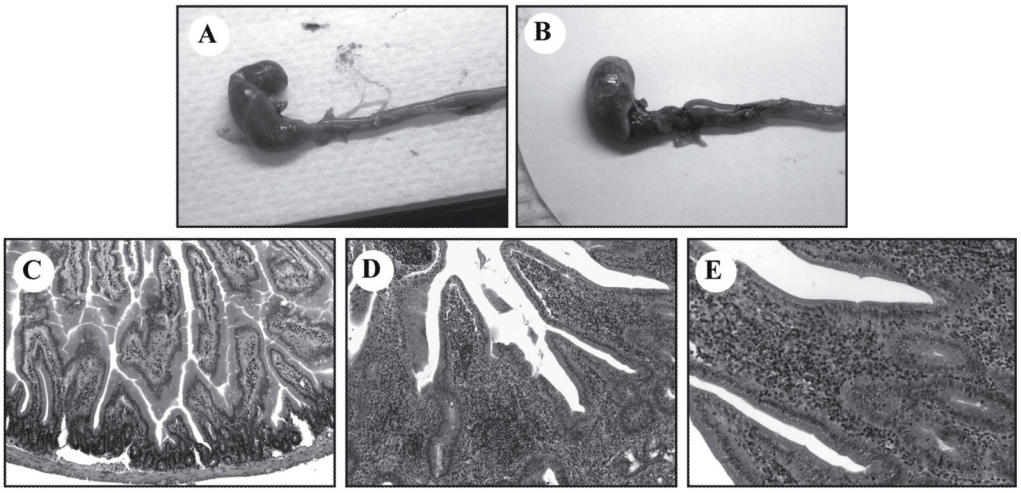


**Figure 2. Extensive inflammation in the duodena of *B. animalis* monoassociated IL-10−/− mice**

Stomach through proximal small intestine was excised from WT (A) and IL-10−/− (B) mice monoassociated with *B. animalis* for 23 weeks. Duodenal thickening and partial obstruction was noted in IL-10−/− animals. H & E stained paraffin sections revealed healthy WT duodenal tissue (C) and severe inflammation in the duodenum of an IL-10−/− mouse (D–E), characterized by massive cellular infiltration and loss of villus architecture. C and D are at 10X magnification and E is at 20X magnification.

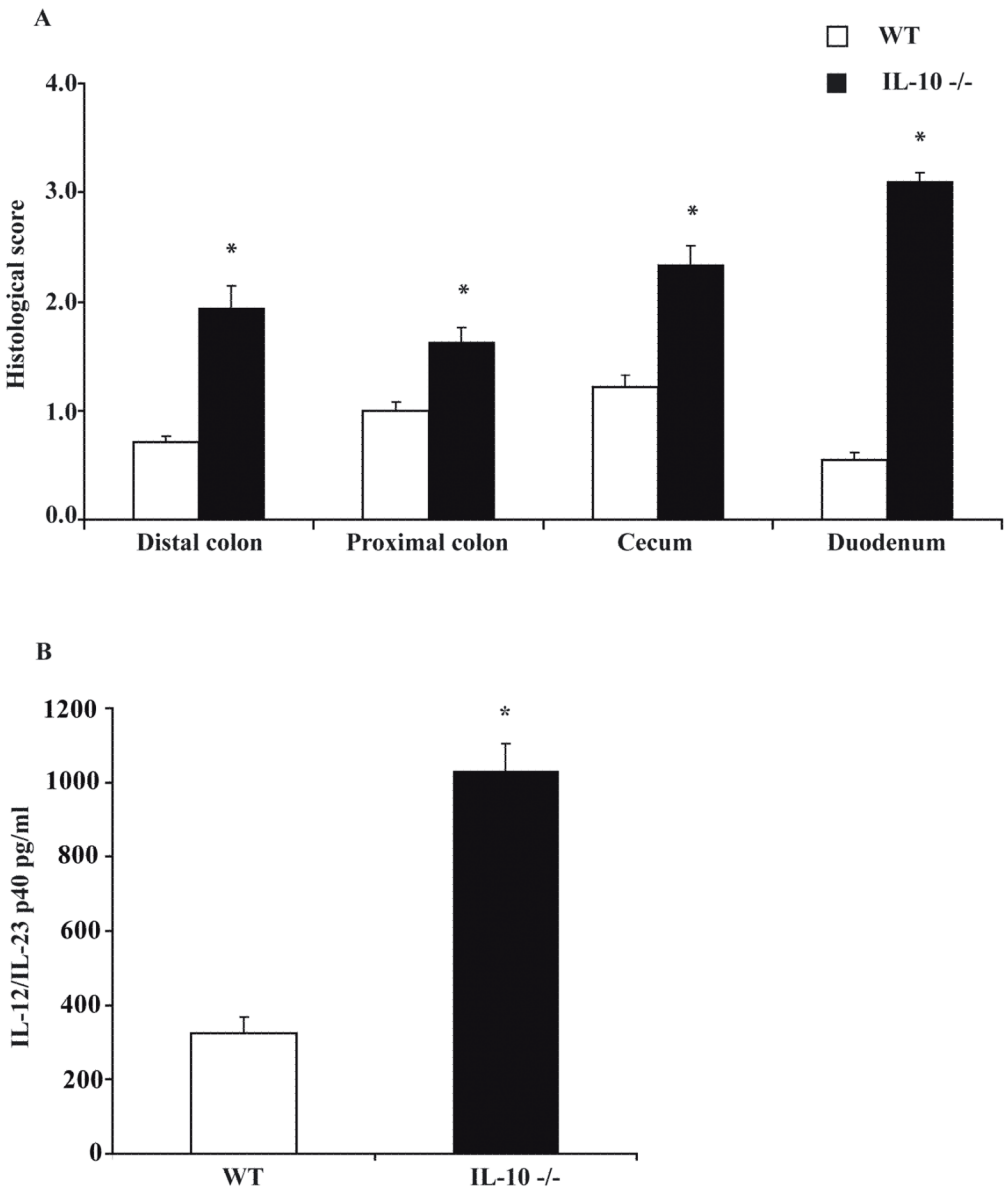


**Figure 3. Quantitative evidence of inflammation in the intestinal tracts of *B. animalis* monoassociated IL-10−/− mice**

(A) Blinded histological scores (mean score ± SE) of distal colon, proximal colon, cecum (n = 19) and duodenum (n = 10 for WT and 16 for IL-10−/−) from WT and IL-10−/− mice after 23 wks of monoassociation with *B. animalis*. (*p<0.01 vs. WT) (B) Spontaneous IL-12/IL-23 p40 secretion was quantified from colonic explant culture supernatants incubated for 20–24 hours. WT mice, n = 18, IL-10−/− mice, n = 19. (*p<0.0001 vs. WT).

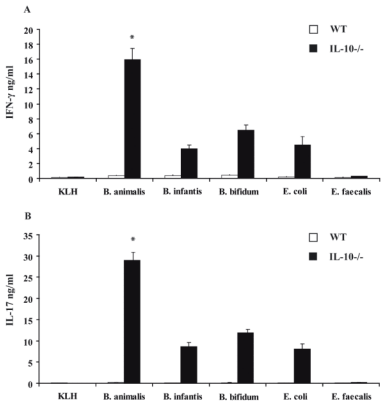


**Figure 4. Bacterial antigen–specific CD4$^+$ T cell responses in the MLN of *B. animalis* monoassociated mice**

CD4$^+$ T cells prepared from mesenteric lymph node (MLN) cells of *B. animalis* monoassociated WT (n = 2–8 mice per stimulator) or IL-10−/− (n = 15–19 mice per stimulator) were co-cultured with WT antigen presenting cells that were pulsed with either KLH ( unrelated antigen control) or with various bacterial lysates *(B. animalis; B. infantis; B. bifidum; E. coli or E. faecalis*). (A) IFN-γ and (B) IL-17 were measured from cell culture supernatants collected at 72 hours. (*p<0.0001 *B. animalis* IL-10−/− vs. *B. infantis*, *B. bifidum*, *E. coli* and *E. faecalis* IL-10−/−).

## TABLE 1

*B. infantis* induces low levels of inflammation in IL-10−/− mice.

| | | WT | IL-10−/− |
|---|---|---|---|
| Histology score (0–4)[a] | distal colon | 0.6 ± 0.0 | 0.7 ± 0.1 |
| | cecum | 0.9 ± 0.1 | 1.3 ± 0.2 |
| | proximal colon | 0.8 ± 0.1 | 1.1 ± 0.2 |
| | duodenum | 0.6 ± 0.1 | 1.5 ± 0.2[c] |
| IL-12/IL-23 p40 (pg/ml)[b] | | | |
| | colonic explant | 158.2 ± 27.0 | 377.6 ± 35.7[c] |

[a] Blinded histological scores (mean score ± SE) of distal colon, proximal colon and duodenum; WT (n = 10) and IL-10−/− (n = 9) mice after 22–23 wks of monoassociation with *B. infantis*.

[b] Spontaneous IL-12/IL-23 p40 secretion (pg/ml) from colonic explants.

[c] Significantly different (*p<0.001 vs. WT).

## TABLE 2

Bacterial cell numbers in the distal colon, proximal colon, cecum and duodenum of *B. animalis* IL-10−/− and WT mice.

| | Distal colon | Proximal colon | Cecum | Duodenum |
|---|---|---|---|---|
| IL-10−/− | 9.4 ± 0.3 | 8.8 ±0.5 | 9.4 ± 0.1 | 6.6 ± 1.4 |
| WT | 9.3 ± 0.5 | 9.0 ± 0.3 | 9.5 ± 0.2 | 6.7 ± 1.3 |

Values represent bacterial counts (mean $\log_{10}$ cfu ± SD per gram tissue).

## TABLE 3

IL-12/IL-23 p40 secretion from WT BMDC stimulated with LPS or bacterial lysates.

| Stimulator | IL-12/IL-23 p40 pg/ml[a] |
|---|---|
| Media | 23.5 ± 0.7 |
| LPS | 13,307.7 ± 1975.9 |
| *B. animalis* | 815.7 ± 76.9 |
| *B. infantis* | 935.0 ± 94.9 |
| *B. bifidum* | 1,012.0 ± 41.6 |
| *E. coli* | 15,307.7 ± 4137.4 |
| *E. faecalis* | 114.7 ± 16.8 |

[a]Values represent IL-12/IL-23 p40 pg/ml (mean ± standard deviation) of triplicate wells. This experiment was repeated with similar results.