A Specific Mutation in Muc2 Determines Early Dysbiosis in Colitis-Prone Winnie Mice

Marina Liso*, MBiol, * Stefania De Santis, PhD, † Giulio Verna, MBiol, * Manuela Dicarlo, PhD, * Maria Calasso, PhD, ‡ Angelo Santino, PhD, ‡ Isabella Gigante, PhD, ‡ Rajaraman Eri, PhD, ‡ Sathuwarman Raveenthiraraj, MSC, Anastasia Sobolewska, PhD, Valeria Palmitessa, PhD, ‡ Antonio Lippolis, MBiol, ‡ Mauro Mastronardi, MD, ‡ Raffaele Armentano, MD, ‡ Grazia Serino, PhD, *° Maria De Angelis, PhD, °, a and Marcello Chieppa, PhD °, ** a, ° , °

Background: Inflammatory bowel disease (IBD), including Crohn disease (CD) and ulcerative colitis (UC), is a multifactorial disorder characterized by chronic inflammation and altered gut barrier function. Dysbiosis, a condition defined by dysregulation of the gut microbiome, has been reported in patients with IBD and in experimental models of colitis. Although several factors have been implicated in directly affecting gut microbial composition, the genetic determinants impacting intestinal dysbiosis in IBD remain relatively unknown.

Methods: We compared the microbiome of normal, uninfamed wild-type (WT) mice with that of a murine model of UC (ie, Winnie strain). Winnie mice possess a missense mutation in Muc2 that manifests in altered mucus production as early as 4 weeks of age, with ensuing colonic inflammation. To better address the potential role of mutant Muc2 in promoting dysbiosis in Winnie mice, we evaluated homozygous mutant mice (Winnie−/) with their WT littermates that, after weaning from common mothers, were caged separately according to genotype. Histologic and inflammatory status were assessed over time, along with changes in their respective microbiome compositions.

Results: Dysbiosis in Winnie mice was already established at 4 weeks of age, before histologic evidence of gut inflammatory changes, in which microbial communities diverged from that derived from their mothers. Furthermore, dysbiosis persisted until 12 weeks of age, with peak differences in microbiome composition observed between Winnie and WT mice at 8 weeks of age. The relative abundance of Bacteroidetes was greater in Winnie compared with WT mice. Verrucomicrobia was detected at the highest relative levels in 4-week-old Winnie mice; in particular, Akkermansia muciniphila was among the most abundant species found at 4 weeks of age.

Conclusions: Our results demonstrate that mutant genetic determinants involved in the complex regulation of intestinal homeostasis, such as that observed in Winnie mice, are able to promote early gut dysbiosis that is independent from maternal microbial transfer, including breastfeeding. Our data provide evidence for intestinal dysbiosis attributed to a Muc2-driven mucus defect that leads to colonic inflammation and may represent an important target for the design of future interventional studies.

Key Words: microbiome, inflammatory bowel disease (IBD), dysbiosis, murine model of ulcerative colitis (UC)

INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn disease (CD) and ulcerative colitis (UC), are multifactorial disorders characterized by chronic bouts of inflammation and remission of the gastrointestinal tract that symptomatically present with abdominal pain, diarrhea, and rectal bleeding.1, 2 Although the precise etiology is currently unknown, it is widely accepted that IBD results from a multifactorial

Author contributions: M.L., S.D.S., G.V., D.M., V.P., S.R., I.G. performed the experiment; M.L., S.D.S., M.Ca., R.A. analyzed the data; M.Ch., M.D.A., A.Sa., G.S., A.So. were involved in the experimental design; M.L., R.E., A.So., M.D.A., M.Ch. edited the manuscript; M.L., R.E., A.So., G.S., M.M., A.L., S.D.S., M.D.A., M.Ch. contributed to scientific discussion.

Address correspondence to: Marcello Chieppa, PhD, National Institute of Gastroenterology “S. de Bellis,” Institute of Research, Castellana Grotte (BA), Italy (transmed@irccsdebellis.it).

© 2019 Crohn’s & Colitis Foundation. Published by Oxford University Press on behalf of Crohn’s & Colitis Foundation.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
doi: 10.1093/ibd/izz279
Published online 21 November 2019

Received for publications March 4, 2019; Editorial Decision October 19, 2019.

From the *National Institute of Gastroenterology “S. de Bellis,” Institute of Research, Castellana Grotte (BA), Italy; †Department of Pharmacy, University of Salerno, Fisciano (SA), Italy; ‡Department of Soil, Plant and Food Sciences, University of Bari, Bari, Italy; §Institute of Sciences of Food Production C.N.R., Unit of Lecce, Lecce, Italy; ¶Mucosal Biology, School of Health Sciences, University of Tasmania, Launceston, TAS, Australia; ‖School of Pharmacy University of East Anglia, Norwich Research Park, Norwich, UK; **Department of Immunology and Cell Biology, European Biomedical Research Institute of Salerno (EBRIS), Salerno, Italy

* Equal contribution

Conflicts of Interest: The authors declare no financial or commercial conflicts of interest.

Supported by: This work was supported by the Italian Ministry of Health GR-2011-02347991, GR-2009-1470633, the Ricerca Corrente 2016 and PON “2014-2020 E FSC –C.P. ARS01_01220” – BIOMIS.
combination of genetic predisposition and environmental factors. In the 20th century, increased urbanization, hygiene practices, air pollution, and widespread use of antibiotics have been reported to contribute to the onset of IBD, particularly in industrialized countries. Epidemiological studies suggest that diets characterized by a high content of animal protein and fats and low fiber and antioxidants sustain the prevalence of bacteria with proteolytic and putrefactive metabolism, particularly on saccharolytic bacteria. Proteolytic bacteria produce toxic metabolites, such as thiols, phenols, indoles, amines, and branched-chain fatty acids, which can lead to reduction of the inner mucus layer, intestinal barrier impairment, loss of immune tolerance, and perturbation of normal gut homeostasis. It is well established that the intestinal microbiome is of paramount importance in the maintenance of gut health. In fact, recent studies underscore its importance for normal intestinal homeostasis by maintaining symbiosis between host and microbial communities that can regulate the fine equilibrium between tolerance and inflammation. However, to date, it is still unclear whether the shift from a “healthy microbiome” to a state of “intestinal dysbiosis” is the result of a chronically inflamed intestine or if dysbiosis triggers gut dysfunction, including dysregulated intestinal permeability and immune response.

Under homeostatic conditions, a dense layer of mucus separates luminal microbial communities from the host’s enteroctyes. While the inner mucus layer is inaccessible to the vast majority of bacterial species, an important interface exists between the gut microbiome and the outer mucus barrier that represent their habitat. It is well established that the intestinal microbiome is of paramount importance in the maintenance of gut health. In fact, recent studies underscore its importance for normal intestinal homeostasis by maintaining symbiosis between host and microbial communities that can regulate the fine equilibrium between tolerance and inflammation. However, to date, it is still unclear whether the shift from a “healthy microbiome” to a state of “intestinal dysbiosis” is the result of a chronically inflamed intestine or if dysbiosis triggers gut dysfunction, including dysregulated intestinal permeability and immune response.

In general, dysbiosis in IBD patients is characterized by a reduction in microbial diversity compared with healthy patients and an overgrowth of specific bacterial species that are usually underrepresented. Importantly, dysbiosis persists even in IBD patients who respond to pharmacological intervention and achieve remission; in fact, it has been proposed that persistent dysbiosis may be the trigger for disease recurrence in these patients. Therefore, it is important to utilize experimental models that mirror the spontaneous and progressive nature of IBD to address the precise timing and specific role(s) of dysbiosis and its onset.

Herein, we evaluated the gut microbiome in a spontaneous model of UC called Winnie and compared that derived from wild-type (WT) mice from 4 to 16 weeks. Both mice strains were obtained as littermates of same Winnie+/− parents. In Winnie mice, the missense mutation of the Muc2 gene causes aberrant MUC2 synthesis and depletion of the mucus layer, resembling that of UC patients. We performed a detailed evaluation of the microbiome and colonic pathology of Winnie and WT control littermates, from weaning to adult age. Our results show that, in contrast to what has previously been described, the onset of specific microbial communities is detectable in 4-week-old Winnie mice, even before the emergence of the intestinal UC-like morphologic features that are characteristic of this strain. Our data indicate that the composition of the gut microbiome is determined by mouse genotype before the onset of colitis, suggesting that dysbiosis observed in the Winnie strain is likely a trigger, more than a consequence, of chronic colonic inflammation in these mice.

METHODS

Ethical Considerations

Our studies were conducted in accordance with national and international guidelines and were approved by the authors’ institutional review board (Organism For Animal Wellbeing [OPBA]). All animal experiments were carried out in accordance with Directive 86/609 EEC, enforced by Italian D.L. n. 116/1992, and approved by the Committee on the Ethics of Animal Experiments of Ministero della Salute–Direzione Generale Sanità Animale (Prot. 768/2015-PR 27/07/2015) and the official RBM veterinarian. Animals were immediately killed if found to be in severe distress during the experimental period to avoid undue suffering.

Mice

C57BL/6 mice were originally purchased from Jackson Laboratories (C57BL/6, Stock No.: 000664), whereas Winnie−/− mice were obtained from the University of Tasmania (Dr. R. Eri’s laboratory). These mouse strains were initially used to generate heterozygote Winnie+/− breeders. For the present study, the breeding strategy was based on co-housing heterozygote breeders to obtain both Winnie−/− and WT littermates from the same mother. Newborn pups were weaned at 4 weeks of age, ear-tagged, and then single-caged based on sex and similar genotype(s) for fecal material collection. Genotype was performed from DNA obtained from 5-mm tail tissues. Body weight, stool consistency, and rectal bleeding were assessed every 4 weeks.

Mice were killed at 16 weeks of age, and colons were removed to evaluate the clinical severity of disease. Colon lengths and weights were measured as indicators of colonic inflammation. Colon/body weight indices were calculated as the ratio of colon wet weight to total body weight (BW) and as the ratio of colon length to total BW of each mouse. Disease Activity Index (DAI) was determined by scoring changes in body weight (0–4), stool consistency (0–4), and occult blood (0–4), as previously described.

Histologic Assessment

Tissue sections from the distal colon were fixed in 10% buffered formalin and embedded in paraffin. Microtome sections of 3 μm were cut and stained using a hematoxylin and eosin.
standard protocol. Images were acquired using a Leica LMD 6500 microscope (Leica Microsystems, Wetzlar, Germany).

**Single-Cell Preparation From Intestinal Lamina Propria and Cytocytotoxicometric Assay**

The intestines of 16-week-old Winnie and WT mice were removed and tissue digested to obtain a single-cell preparation. Colons were cut into small segments (1 cm long), washed with DPBS 1X (Gibco, Waltham, MA, USA) + 2.5 mM EDTA (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) to remove epithelial cells, and digested with collagenase type IV and DNase I (Sigma Aldrich, St. Louis, MO, USA) using the GentleMacs suggested protocol for 30 minutes at 37°C. Resulting single-cell suspensions from colonic lamina propria (LP) were pelleted by centrifugation, washed with DPBS 1X + 0.5 mM EDTA, and passed through 100-µm and 30-µm cell strainers (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then washed with DPBS 1X + 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) and labeled with CD45.2-FITC and MHC II-APC (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. Finally, cell suspensions were labeled with 7-AAD Staining Solution (Miltenyi Biotec, Bergisch Gladbach, Germany) to distinguish between viable and dead cells, also according to the manufacturer’s instructions. Flow cytometer data analysis was performed using NAVIOS software (Beckman Coulter, Brea, CA, USA), with at least 3 experiments performed.

**RNA Extraction and Quantitative Polymerase Chain Reaction Analysis**

Total RNA was isolated from colons of WT and Winnie mice. RNA was extracted using TRIzol (Thermo Fisher Scientific, MA, USA), according to the manufacturer’s instructions. Total RNA (1 µg) was reverse-transcribed using an iScript cDNA Synthesis kit (Biorad, CA, USA) with random primers for cDNA synthesis. Gene expression of Ptprc (CD45) and Gapdh was assessed using the TaqMan gene expression assay (Thermo Fisher Scientific, MA, USA) murine probes Mm01293577_m1 and Mm99999915_g1, respectively. Real-time analysis was performed on a CFX96 System (Biorad, CA, USA), and relative expression was calculated using the ΔΔCt method. At least 3 experiments were performed.

**DNA Extraction From Fecal Material**

Total genomic bacterial DNA was isolated from frozen stool samples of 4-, 8-, and 16-week-old WT and Winnie mice (4 samples/genotype for each time point) using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions.

16S rRNA Metagenetics of Gut Microbiome

16S metagenomics was carried out at Genomix4life (Salerno, Italy) using the Illumina MiSeq platform. The V3-V4 region of 16S bacterial rRNA was amplified for analysis of diversity. Polymerase chain reaction (PCR) and sequencing analyses were carried out according to standard protocols of Genomix4life. Quality control (QC) and taxonomic assignments were performed according to the QIIME and Ribosomal Database Project Bayesian classifier in combination with a set of custom-designed informatic pipelines implemented by Genomix4life for analyses of microbial communities. Taxonomic attribution was carried out using the BLAST search in the NCBI 16S ribosomal RNA sequences database. The percentage of each bacterial OTU was analyzed individually for each sample, providing relative abundance information among the samples based on the relative numbers of reads within each sample. Alpha-diversity indexes were evaluated using the number of OTUs, Chao1 species richness, and the Shannon index. Alpha diversity was calculated using QIime.

**Statistical Analysis**

All data are expressed as the mean ± SEM, with results obtained from 3 independent experiments. Metagenomic data (Unifrac distance metric and taxonomic abundance) were analyzed by principal component analysis (PCA) using the statistical software Statistica for Windows (Statistica 6.0 for Windows 1998, StatSoft, Vigonza, Italia). Permut-MatrixEN software was used to identify clusters at the level of mouse groups and taxa. Statistical analysis of relative abundance of microbial genera was based on Duncan’s Multiple Range test, with a significance level of P ≤ 0.05. Finally, unless specifically described, other data and group differences were analyzed and compared by paired or unpaired 2-tailed Student t tests.

**RESULTS**

**Body Weight Loss During Colitis Depends on Genotype Predisposed to Develop UC-Like Symptoms**

Experimental mice were obtained from heterozygote breeders (Winnie+/-) to obtain Winnie-/- and WT mice from the same breeding pair(s). Weight differences were associated with the defect in Muc2 that characterizes Winnie mice as early as 4 weeks of age, which persisted over the course of time up to 16 weeks of age. Although WT mice consistently gained weight over the course of the experimental period (~50% of the initial body weight from 4 to 16 weeks), both Winnie-/- males and females gained less weight than their WT counterparts, reaching 23.2 g in males and 18.9 g in females from 4 to 16 weeks of age (Fig. 1).
Moderate Inflammation in the Intestine of 16-Week-Old Winnie Mice

We next compared the inflammatory status of WT and Winnie littermates obtained from the same breeders. As observed in previous studies, weight loss in 16-week-old Winnie mice correlated with the presence and severity of colonic inflammation. Loss of stool consistency resulting in more watery stools was also observed only in Winnie mice (Fig. 2A), which also showed a reduction in colon length, although this did not reach statistical significance (Fig. 2B). The colon weights of Winnie mice were significantly increased compared with age-matched WT mice (Fig. 2C), suggesting the presence of inflammation. Consequently, colon length/mouse weight and colon weight/mouse weight indices were also significantly increased when comparing Winnie with WT mice (Fig. 2B). Histologic evaluation of intestinal tissues confirmed the presence of moderate colitis, with epithelial erosions and immune cell infiltration in 16-week-old Winnie mice, as previously reported (Fig. 2F).

Increased Immune Cell Infiltration in LP of 16-Week-Old Winnie Compared With WT Mice

Fluorescence-activated cell sorting analysis revealed a significant increase in the percentage of CD45.2+ cells in Winnie compared with age- and sex-matched WT mice. Gating on CD45.2+ MHC II+ and CD45.2+ MHC II- viable cells showed that both of these populations were present in greater numbers in Winnie colons compared with WT (Fig. 3A), with CD45.2+ MHC II+ cells increased from 3.9% in WT to 7.9% in Winnie (P = 0.021), whereas CD45.2+ MHC II- cells increased from 1.8% in WT to 6.6% in Winnie (P = 0.020). Furthermore, a 4-fold increase in relative mRNA expression of Cd45 was present in the colons of Winnie compared with WT mice (Fig. 3B), while Cd45 and MhcII were below the detection limit in both 5-week-old WT and Winnie mice (data not shown).

Age-Dependent Microbiome Composition Is Distinct in Winnie vs WT Mice

We recently demonstrated that the intestinal microbiome of adult Winnie mice is clearly distinguishable from WT mice.

However, it is unclear if dysbiosis in Winnie mice is the result of colitis progression or if dysbiosis itself drives ensuing colonic inflammation. As such, we analyzed the microbiome of
FIGURE 2. Aberrant morphologic and histologic features in colons from Winnie vs WT mice. Representative images of whole colons (A) and measurements of colon length (B) and colon weight (C) of 16-week-old Winnie and WT male mice. D and E, Colon length/mouse weight and colon weight/mice weight indices, respectively. F, Hematoxylin and eosin staining of distal colon sections shows abundant mucus-secreting goblet cells with moderate inflammation only in 16-week-old Winnie mice and evident signs of erosion within the intestinal epithelium compared with WT controls. Scale bar = 100 µm. No signs of inflammation are present in 4- and 8-week-old mice. ***P < 0.001; *P < 0.05 (Student t test).
Winnie mice at different ages, from time of weaning to adult age, and compared the results obtained from WT mice to assess if dysbiosis was either the cause or effect of intestinal inflammation in this unique mouse strain.

Experimentally, we evaluated the microbiome composition in 4-, 8-, and 16-week-old WT vs Winnie mice. Compared with WT, Winnie mice showed the highest ($P < 0.05$) Shannon index value at 4 weeks of age (Supplementary Table 1). A similar trend was also observed at 8 and 16 weeks. The highest values of OTUs and Chao1 were evident in Winnie samples compared with WT. The only exception was for Chao1, which did not differ ($P > 0.05$) at 4 weeks of age. Three phylogeny-based $\beta$-diversity analyses, using the Unifrac distance metric, showed clustering of samples according to genotype (WT or Winnie mice) (Supplementary Fig. 1). Within the same genotype, samples taken at 4 weeks of age were clearly separate from the others (8 and 16 weeks), especially for WT samples.

In regards to the analysis based on phylum, abundance of Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia, Tenericutes, Deferrribacteres, and Actinobacteria represented $>99\%$ of all 16S rDNA sequenced. Bacteroidetes and Firmicutes were the most representative phyla for both WT and Winnie mice. The relative abundance of Bacteroidetes was higher in Winnie compared with WT mice, particularly at 8 weeks of age. Firmicutes, however, did not differ between the 2 genotypes. In this regard, compared with 4 weeks of age, the relative amount of Firmicutes increased after 8 and 16 weeks of age for WT and Winnie, respectively (Fig. 4). The relative amount of Proteobacteria significantly differed among samples, especially for WT and Winnie at 16 weeks (17.85 vs 8.01%, respectively; $P = 0.011$). Verrucomicrobia were found at the highest relative levels in 4-week-old Winnie mice, after which relative abundance decreased with age. On the contrary, Deferrribacteres was significantly lower in Winnie compared with WT samples.

In addition, compared with WT, Winnie mice showed the highest ($P < 0.05$) relative amount of Alkaliphilus, Candidatus Blochmannia, and Caldilinea genera in all weeks analyzed (Fig. 5). On the other hand, the presence of Helicobacter, Odoribacter, and Lactobacillus were mainly associated with WT samples. At 4 weeks, Winnie mice showed 3 other genera (Dysgonomonas, Olivibacter, and Rubritalea), which were all present in greater abundance than in WT mice. Compared with 4 weeks, the greatest differences in the profiles of genera were found in 8-week-old mice. Bacteroides, Parabacteroides, Porphyromonas, Clostridium, Paraprevotella, Acholeplasma,
Butyricimonas, and Desulfotomaculum were more abundant (\(P < 0.05\)) in Winnie compared with WT mice in 16-week-old mice and especially in 8-week-old mice. In contrast, Blautia, Mucispirillum, and Sedimentibacter were found to be more abundant in WT samples and increased in 8- and/or 16-week-old mice. Furthermore, we observed that the relative abundance of most bacterial genera changed with age, from 4 to 16 weeks of age. Surprisingly, the tendency to become more or less represented was similar for the vast majority of genera, independent from the host genotype. In particular, Figure 5 shows an increased presence of the Mucispirillum and Caldilinea genera in both experimental groups, whereas Odoribacter and Bacteroides decreased with age. Some bacterial genera, instead, showed an opposite tendency between the
Early Dysbiosis in Winnie Mice

2 genotypes. For example, *Parabacteroides* and *Lactobacillus* increased from 4 to 16 weeks of age in *Winnie* mice, but decreased in WT mice. This age-dependent tendency for decreased *Lactobacillus* in WT mice has been previously reported by Vemuri et al.29

Finally, in regards to species, the relative abundance of 62 species differed between samples (Fig. 6; Supplementary Table 2). Compared with WT, *Winnie* mice showed 9 bacterial species (*Akkermansia muciniphila, Alkaliphilus crotonotaxidans, Caldilinea tarbellica, Clostridium caenicola, Clostridium thermosuccinogenes, Desulfitobacterium autotrophicus, Luteolibacter algae, Porphyromonas circumdentaria, and Tindalia magadiensis*) that were more abundant at 4 weeks of age (*P* < 0.05). All of the aforementioned species showed a
relative abundance that was always greater in Winnie than WT mice at 8 and 16 weeks of age. The highest differences in species profile (39 OTUs; \( P < 0.05 \)) between genotypes were found in 8-week-old mice.

**DISCUSSION**

In this study, we compared the fecal microbiome of littermates (WT and Winnie mice) from the same mother(s) at 4, 8, and 16 weeks. Newborn pups were weaned immediately after the 4-week fecal material collection. The results of our study demonstrate that, even if delivered from the same mother and fed the same milk containing identical antibody repertoires, the specific genotype was the driving force for the unique microbiome footprint associated with either WT or Winnie mice.

Dysbiosis of the intestinal microbiome associated with chronic gut inflammation has become a major area of interest and research for IBD investigators. Abundance of microbial species seems to be a reliable indicator of IBD outcome; at the same time, persistence of intestinal dysbiosis can lead to disease recurrence, even in patients who successfully achieve remission. Maternal transmission has been indicated as the pivotal event for the onset of colonization of intestinal communities; for this reason, we investigated if the differences observed between the fecal microbiome of WT and Winnie littermates could still be observed in offspring from the same parents.

The Winnie murine model is a model of spontaneous and progressive UC displaying certain morphologic and microbial characteristics, which has been determined using conventional breeding approaches. Initially generating heterozygote breeders (Winnie\(^{+/+}\)) derived from C57BL/6 and homozygous Winnie mutant mice, we evaluated resulting littermate pups from the same mother(s). We first assessed the onset of the progressive UC-like colitis in the Winnie model by morphologic and histologic features, along with immune cell infiltrate of the large intestine. Strikingly, our fecal microbiota metagenomic analysis shows that Winnie offspring displayed significant dysbiosis as early as 4 weeks of age compared with their WT siblings/littermates the same age, which is similar to what has been reported at 12 weeks of age.\(^{30}\) Overall, our data support a more dominant role for the host genotype in conferring dysbiosis onset compared with microbial transmission from the mother at birth. Of note, the possibility exists that analysis of microbiota derived from fecal samples may be more relevant if focused on mucus-associated bacteria present within the colon. However, due to the experimental design based on prospective microbiota analysis, it was not possible to collect samples from the same individual mouse at 4, 8 and 16 weeks.

An abundance of microbial species perfectly clustered, depending on host expression of the Winnie mutation, with the greatest significance at 8 weeks after weaning, when the immune machinery of the host is developing its immunoglobulin repertoire.\(^{31}\) Bacteroidetes were mainly associated with the Winnie genotype. Previously, it was shown that Bacteroidetes establish a crucial interaction with the host at an early stage of life, and their colonization in germ-free mice affects immune system development.\(^{32}\) Indeed, Bacteroides and Prevotella, 2 of the most abundant genera in the intestinal microbiome within the Bacteroidetes phylum, are associated with UC.\(^{33}\) In contrast, a previous meta-analysis reported 9 studies (706 patients) published between 2002 and 2012 showing that the mean level of Bacteroides was significantly lower in CD and UC patients, especially in the active phase of disease.\(^{34}\) The differences in Bacteroides may be assigned to individual species.

We also found that several species belonging to different genera (Bacteroides, Parabacteroides, Porphyromonas, Paraprevotella, and Sphingobacterium) of Bacteroidetes were mainly associated with the Winnie genotype, particularly in 8-week-old mice. The only 2 Bacteroidetes species reduced in Winnie compared with WT mice were Odoribacter denticanis and Rikenella microfusus. The controversial role of Bacteroidetes in the development of inflammatory intestinal diseases will be the focus of future studies in our lab and raises the possibility of promising therapies targeting mucosa-associated bacterial composition.

Within Firmicutes, some species (eg, Alkaliphilus crotonatoxidans, Clostridium alcalophilum) were associated with the Winnie genotype. On the contrary, other Firmicutes (eg, Blautia cocoides, Oscillospira eae, Ruminococcus gnavus) were less represented in Winnie compared with WT mice. Previously, dogs with chronic inflammatory enteropathy showed a lower amount of Blautia and Ruminococcus compared with healthy dogs.\(^{35}\) R. gnavus showed a positive role in the intestinal microbiota, producing an antibacterial peptide that protects hosts against pathogens.\(^{36}\)

Several studies have evaluated the influence of different genes on the intestinal microbiome and showed that NLRP6, an innate immune receptor, is important in suppressing the development of spontaneous colitis in IL10\(^{−/−}\) mice.\(^{37}\) Moreover, NLRP6 deficiency induces enrichment of Akkermansia muciniphila that can act as a pathobiont to promote colitis in genetically susceptible hosts. In this study, Akkermansia muciniphila was found to be more abundant in Winnie compared with WT mice at the time of weaning (4 weeks of age). This finding is somewhat surprising, as in IBD patients a decrease of this species has been observed.\(^{38}\) Akkermansia muciniphila is a mucin-degrading bacterium; thus, in Winnie mice characterized by a compromised and less compact inner mucus layer, mucin availability could favor Akkermansia muciniphila outgrowth.\(^{39, 40}\) Indeed, Winnie mice are different from Muc2 deficient mice, which are also characterized by acute spontaneous colitis and colorectal cancer development.\(^{12, 41}\) MUC2 is present in Winnie, but is not firmly compacted in a tight inner layer.\(^{17}\) It is important to note that Akkermansia muciniphila abundance is reduced in older mice, when the inflammatory response becomes more evident (\( P = 0.008 \) in 4- vs 8-week-olds). These findings are in line with what has been previously reported.\(^{27}\) Indeed, Schneeberger and colleagues correlated levels of...
Early Dysbiosis in Winnie Mice

Akermannia muciniphila with inflammatory markers and showed that these were inversely correlated. 42

Interestingly, we found that the Helicobacter genus is mainly associated with WT mice, with *H. masto myr us* as a dominant species. *H. masto myr us* colonizes the liver of mice, but the pathogenic potential of this bacterium is unknown. 43 Indeed, the role of Helicobacter species in IBD is not yet fully understood and is somewhat controversial. *Helicobacter pylori* infection is negatively associated with IBD, regardless of ethnicity, age, and previous use of aminosalicylates and corticosteroids with antibiotics, influencing the magnitude of this association. Closely related bacteria, including Enterobacterial Helicobacter spp., (EHS) and Campylobacter spp., can also increase the risk of IBD. 43 It has also been suggested that *H. pylori* might exert an immunomodulatory effect in IBD. 44

CONCLUSIONS

Overall, using the Winnie model of colitis, we demonstrate that alterations of genes, such as the Muc2 mutation in this mouse strain, which impacts the maintenance of intestinal homeostasis, may result in early intestinal dysbiosis that is independent from maternal microbial transfer, including breastfeeding. Once established, differences between microbial communities persist and continue with age. Results from the present study were derived using heterozygote mothers; as such, future studies using mothers with different genetic backgrounds will be studied to further elucidate the influence of maternal microbial transfer on the offspring’s microbiome. Our study has aided in providing a map of intestinal dysbiosis dependent on mutagenesis of Muc2 that may be useful for future targeted interventionist studies.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

ACKNOWLEDGMENTS

We are grateful to all members of LAB-81 and to Genomix4life for their constructive help and support.

REFERENCES

1. Corridoni D, Arsenneau KO, Connelli F. Inflammatory bowel disease. *Immunol Lett.* 2014;161:231–235.
2. Ungaro R, Mehandru S, Allen PB, et al. Ulcerative colitis. *Lancet.* 2017;389:1756–1770.
3. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol.* 2003;3:211–233.
4. Luo Y, de Lange KM, Jostins L, et al. Exploring the genetic architecture of inflammatory bowel disease by whole-genome sequencing identifies association at ADCY7. *Nat Genet.* 2017;49:186–192.
5. McGovern DP, Kugathasan S, Cho JH. Genetics of inflammatory bowel diseases. *Gastroenterology.* 2015;149:1163–1176.e2.
6. Ananthakrishnan AN, Bernstein CN, Illopooulus D, et al. Environmental triggers in IBD: a review of progress and evidence. *Nat Rev Gastroenterol Hepatol.* 2018;15:39–49.
7. Kronman MP, Zaatous TE, Haynes K, et al. Antibiotic exposure and IBD development among children: a population-based cohort study. *Pediatrics.* 2012;130:e794–e803.
8. Ananthakrishnan AN. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol.* 2015;12:205–217.
9. Basson A, Torel A, Rodriguez-Palacios A, et al. Mucosal interactions between genetics, diet, and microbiome in inflammatory bowel disease. *Front Immunol.* 2016;7:290.
10. Montemurro E, Cosola C, Dalfino G, et al. What would you like to eat, Mr CKD Microbiota? A Mediterranean diet, please! *Kidney Blood Press Res.* 2014;39:114–123.
11. Deau MS, Seekatz AM, Koropatkin NM, et al. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell.* 2016;167:1339–1353.e21.
12. Johansson ME, Phillipson M, Petersson J, et al. The inner of the two Muc2 mucin-dependent mucous layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A.* 2008;105:15064–15069.
13. Yu LC. Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. *J Biomed Sci.* 2018;25:79. doi:10.1186/s12929-018-0483-8
14. Zhang SL, Wang SN, Miao CY. Influence of microbiota on intestinal immune system in ulcerative colitis and its intervention. *Front Immunol.* 2017;8:1674. doi:10.3389/fimmu.2017.01674
15. Sommieri HK, Kugathasan S. The microbiome in patients with inflammatory diseases. *Clin Gastroenterol Hepatol.* 2019;17:243–255.
16. Durchschmied F, Petritsch W, Hammer HF. Diet therapy for inflammatory bowel diseases: the established and the new. *World J Gastroenterol.* 2016;22:2179–2194.
17. Heathwood CK, Cook MC, Eri R, et al. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med.* 2008;5:e54. doi:10.1371/journal.pmed.0050054
18. Chen L, Zhou Z, Yang Y, et al. Therapeutic effect of imiquimod on dextran sodium-sulfate-induced ulcerative colitis in mice. *PLoS One.* 2017;12:e0186138. doi:10.1371/journal.pone.0186138
19. Kindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diagnostic studies. *Nucleic Acids Res.* 2013;41:e1. doi:10.1093/nar/gks808
20. Autschal SF, Gish W, Miller W, et al. Basic local alignment search tool. *J Mol Biol.* 1990;215:403–410.
21. Andreotti R, Pérez de León AA, Dowd SE, et al. Assessment of bacterial diversity in the cattle tuck *Rhizophostus (Boophilus) microplus* through tag-encoded pyrosequencing. *BMC Microbiol.* 2011;11:6. doi:10.1186/1471-2180-11-6
22. Chao A, Bunge J. Estimating the number of species in a stochastic abundance model. *Biometrics.* 2002;58:531–539.
23. Shannon CE, Weaver W. *The Mathematical Theory of Communication.* Urbana, IL: University of Illinois Press; 1949.
24. De Angelis M, Piccolo M, Vannini L, et al. Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PLoS One.* 2013;8:e67993. doi:10.1371/journal.pone.0067993
25. Serrano M, Mache E, Gres S, et al. Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. *Gut.* 2016;65:543–553.
26. Eri RD, Adams RJ, Tran TV, et al. An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity. *Mucosal Immunol.* 2011;4:354–364.
27. De Santis S, Kunde D, Galleggiante V, et al. TNFα deficiency results in increased IL-1β in an early onset of spontaneous murine colitis. *Cell Death Dis.* doi:10.1038/cddis.2017.397
28. Lino M, De Santis S, Scaroni A, et al. A bronze-tomato enriched diet affects the intestinal microbiome under homeostatic and inflammatory conditions. *Nutrients.* 2018;10:E1255. doi:10.3390/nu10091255
29. Vemuri R, Shinde T, Gundamaraju R, et al. *Lactobacillus acidophilus DDS* modulates the gut microbiota and improves metabolic profiles in aging mice. *Nutrients.* 2018;10:E2155. doi:10.3390/nu10091255
30. Robinson AM, Gondalva SV, Karpe AV, et al. Fecal microbiota and metabolome in a mouse model of spontaneous chronic colitis: relevance to human inflammatory bowel disease. *Inflamm Bowel Dis.* 2016;22:2767–2787.
31. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell.* 2014;157:121–141.
32. Gibino G, Lopetuso LR, Scaldaferrì F, et al. Exploring Bacteroidetes: metabolic key points and immunological tricks of our gut commensals. *Dig Liver Dis.* 2016;48:530–539.
33. Lucas K, Mielnik S, Jacobs E, et al. Prevalence of *Bacteroides* and *Prevotella* spp. in ulcerative colitis. *J Med Microbiol.* 2006;55:617–624.
34. Zhou Y, Zhu F. Lower level of bacteroides in the gut microbiota is associated with inflammatory bowel disease: a meta-analysis. *Biomed Res Int.* 2016;2016:5828959. doi:10.1155/2016/5828959.
35. AlSha waqfeh MK, Wajid B, Minamoto Y, et al. A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy. *FEMS Microbiol Ecol.* 2017;93. doi:10.1093/femsec/fix136.

36. Dabard J, Broidonneau C, Philippe C, et al. Ruminococcin A, a new lantibiotic produced by a *Ruminococcus gravis* strain isolated from human feces. *Appl Environ Microbiol.* 2001;67:4111–4118.

37. Serregin SS, Golovchenko N, Schaf B, et al. NLRP6 protects *Il10-/-* mice from colitis by limiting colonization of *Akkermansia muciniphila*. *Cell Rep.* 2017;19:733–745.

38. Png CW, Lindén SK, Gilshenan KS, et al. Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *Am J Gastroenterol.* 2010;105:2420–2428.

39. Berry D, Stecher B, Schintlmeister A, et al. Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing. *Proc Natl Acad Sci U S A.* 2013;110:4720–4725.

40. Van Herreweghen F, Van den Abbeele P, De Mulder T, et al. In vitro colonisation of the distal colon by *Akkermansia muciniphila* is largely mucin and pH dependent. *Benef Microbes.* 2017;8:81–96.

41. Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology.* 2006;131:117–129.

42. Schneeberger M, Everard A, Gómez-Valadés AG, et al. *Akkermansia muciniphila* inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. *Sci Rep.* 2015;5:16643. doi:10.1038/srep16643

43. Castaño-Rodríguez N, Kaakoush NO, Lee WS, et al. Dual role of *Helicobacter* and *Campylobacter* species in IBD: a systematic review and meta-analysis. *Gut.* 2017;66:235–249.

44. Shen Z, Xu S, Dewhirst FE, et al. A novel enterohepatic *Helicobacter* species ‘*Helicobacter mastomyrinus*’ isolated from the liver and intestine of rodents. *Helicobacter.* 2005;10:59–70.