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Original article

COLON MICROBIAL COMPOSITION IS CORRELATED WITH THE SEVERITY OF COLITIS INDUCED BY 2,4,6-TRINITROBENZENESULFONIC ACID IN MICE

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Summary

The objective of this study was to evaluate the changes in some bacterial species of colonic microbiota, the clinical signs and the intestinal changes in mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis.

CD-1 male mice were randomly divided into three groups and inoculated intrarectally with saline, ethanol or TNBS solutions. Ethanol and TNBS treatments induced weight loss accompanied by mild and severe inflammation of the colon mucosa, respectively. However, TNBS-treated mice displayed significant differences compared to the saline group in terms of disease activity index and histological scoring. Both ethanol and TNBS groups showed an increased prevalence of *Escherichia coli* and *Clostridium* spp., a decrease in Lactobacilli and Bifidobacteria counts, as well as changes in the relative proportions of bacteria in the colon.

The results confirm the validity of TNBS treatment to study the mechanisms involved in the pathogenesis and progression of inflammatory bowel diseases (IBD) in CD-1 mice. Gut microbiota may become a diagnostic biomarker with therapeutic potential for IBD in the future.

Introduction

Inflammatory bowel diseases (IBD) are chronic, recurrent and multifactorial conditions affecting the gastro-intestinal tract. Although the aetiology of IBD is still not fully understood, it involves a complex interaction between genetic, luminal and environmental factors, including diet, cigarette smoke and drug exposure, infections, geography and stress, that trigger an inappropriate mucosal immune response. Changes in the composition of intestinal microbiota and an abnormal immune response to gut mi-

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croorganisms are likely to be the key factors in the onset and progression of IBD. IBD include ulcerative colitis (UC) and Crohn's disease (CD), which can be distinguished by the localization of the inflammatory changes in the digestive system, i.e. the portion of the intestinal wall affected by inflammation and microbial perturbations. UC is confined to the colon, while CD may affect any part of the digestive tract. The implications of microbial involvement or causality in IBD are still unclear [1]. The complex microbial population of the intestinal tract plays an important role in host nutrition and health, since different bacterial species establish a rich interaction network involving mutualism, symbiosis and pathogenicity [2]. Colonization occurs mainly in the colon that typically harbors more than 500 different species of bacteria [3]. The microbiota exerts various physiological functions, including: i) inhibition of pathogen growth; ii) mucosal barrier function; iii) synthesis of compounds useful for the trophism of colon mucosa, such as butyric acid, from unabsorbed carbohydrates; iv) modulation of intestinal innate and adaptive immune system; and v) synthesis of several nutrients, such as amino acids and vitamins B and K, and mineral absorption. In healthy subjects, specific mechanisms regulate the immunological tolerance of the host to intestinal microbiota and their metabolic products. The interaction between the host and specific bacterial strains can induce a tolerogenic response to the intestinal microflora through production of regulatory T cells (Treg) and IgAs, suppression of cellular pathways related to the synthesis of pro-inflammatory cytokines, and, finally, production of anti-inflammatory cytokines and antimicrobial peptides [4].

On the other hand, an abnormal intestinal flora is responsible for a large number of negative effects in the host. There is mounting evidence that microbiota can induce conditions like IBD, obesity, and type I diabetes [5]. Resident bacteria play an important role in initiating and perpetuating intestinal inflammation in IBD. It is well known that the quality and quantity of intestinal microbiota vary with disease; some bacterial species may promote the development of a specific disease, while concurrently protecting the host from another disorder. Moreover, the mechanistic potential of bacterial species may also differ between the various parts of the gut. Indeed, there are evidence that intestinal microbiota is greatly different in healthy individuals compared to UC and CD patients. Furthermore, to date, the various studies carried out on IBD have not managed to identify a single specific bacterial species as a cause for this disease [6]. Numerous studies performed in human and animal models have investigated changes in the composition of microbiota in several gastro-intestinal inflammatory diseases, including IBD, using different approaches [7,8]. Currently, different types of methods are being employed for analyzing gut microbiota composition, including bacterial cultures (conventional microbiological techniques), and the most recent and sophisticated molecular biological methods, such as fluorescence *in situ* hybridization, terminal restriction fragment length polymorphism, and metagenomics approaches [9]. Cultivation-based analysis is the most simple and inexpensive way to quantify gut microbiota. While this technique does not allow the detection of some species, it can be used to evaluate physiological parameters, and it does not require extensive bio-informatic analyses. IBD patients present a reduction in biodiversity and a depletion of some bacterial phyla, observed in feces and mucosa-associated microbiota [9]. Specifically, several authors have reported a reduction in the relative abundance of "beneficial bacteria", such as Bifidobacteria and Lactobacilli, and an increase in potentially dangerous bacteria, such as *E. coli*, in gut inflammatory diseases, including IBD [10,11]. Nevertheless, at the moment it is unclear whether dysbiosis is a cause or a consequence of IDB. To understand the pathogenesis of IBD and identify potential therapeutic agents, several chemical- and hapten-induced colitis models have been used, including the TNBS-induced colitis model [12]. This is an experimental model of intestinal inflammation that induces IBD-like histo-

logical and biochemical features. In particular, TNBS in mice closely mimics human IBD both histopathologically [13] and clinically [14]. It is currently thought that intestinal inflammation is induced by two-step process. Firstly, ethanol impairs epithelial barrier functions; TNBS then haptenizes intestinal antigens and microbial proteins, triggering the host immune system. Moreover, intestinal microbiota dysbiosis related to TNBS-induced enterocolitis has been found in zebrafish [15] and rat [16] IBD-like models. However, the effects of TNBS treatment on the colon microbiota of CD-1 mice still need to be elucidated.

The objective of this study was to evaluate the changes in some bacterial species of colonic microbiota in relation to the clinical signs and intestinal changes induced by TNBS in CD1 mice.

Materials and Methods

Animals

Twenty-seven CD-1 strain male mice aged 5 to 6 weeks (weighing 26-34g) were acquired from Harlan Laboratories S.r.l. (Correzzana D'Adda, Milan, Italy). Mice were housed in a controlled environment in terms of temperature (22°C) and photoperiods (12:12-hour light/dark cycle), and allowed unrestricted access to standard mouse chow and tap water. After 10 days of acclimatization, mice were randomly divided into three groups (n=9 per group) and inoculated intrarectally with saline (Saline group), 50% ethanol (Ethanol group), or TNBS solution (TNBS group). All experiments conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Animal care was in compliance with current Italian regulations (Ministerial Declaration 116/92) and the European Economic Community rules (O.J. of European Commission L 358/1 12/18/1986). The experimental design was approved by the Ethical Committee for Animal Experimentation of the University of Perugia, Italy.

Induction of inflammation

Mice were lightly anesthetized with isoflurane (Merial, Milan Italy) and TNBS (1.5 mg/mouse) dissolved in 50% ethanol was administered intrarectally via a catheter (2 biological instruments, Basozzo, Varese, Italy) equipped with a 1-m/ syringe. The catheter was inserted about 3 cm into the rectum, and a total volume of 150 µl TNBS was administered. In order to distribute the TNBS within the colon, the mouse was held in a vertical position with the head down for 1 min following the injection. Mice from the other groups were treated with the same procedure and an equivalent volume of saline or ethanol. Mice were sacrificed 3 days after TNBS administration. Body weight (BW) was measured at TNBS/ saline/ethanol treatment (T0) and at the end of the experimental period (T1)

Determination of disease activity index (DAI)

DAI was determined using the method described by Murano et al. [17], combining weight loss, stool consistency and bleeding scores. BW changes were calculated as the difference between BW at T1 and T0. Diarrhoea was assessed daily through the presence or absence of faecal material adhering to anal fur and confirmed during autopsy through the presence or absence of faecal pellet in the rectum. A 4-point scale was used, with 0 corresponding to a normal faecal pellet and 4 corresponding to frank diarrhoea. Rectal bleeding was evaluated daily through the presence or absence of visible blood in the faecal material, and during autopsy through the presence or absence of gross colonic or rectal bleeding, and scored as 0 for negative and 4 for gross bleeding.

Tissue processing

All surviving mice were euthanized by cervical dislocation 3 days after the intrarectal treatment. At autopsy, a gross evaluation of the digestive tract was carried out. The intact gastrointestinal tract was immediately excised from anus to oesophagus. The large bowel was subdivided into caecum, colon and rectum. Colon was opened longitudinally. The luminal materials were removed, immediately placed into an anaerobic chamber and dissolved in sterile pre-reduced PBS for the bacteriological assays. Colon tissues were cleaned with saline and several samples were collected for the histological evaluation and fixed in a 10%

buffered formalin solution.

Histology

After an overnight fixation in formalin, tissues were dehydrated in alcohol and cleared by xylene before embedding them in paraffin wax. 4-5 µm sections were stained with hematoxylin and eosin (H&E) to evaluate structural and morphological colon alterations.

Histological scoring

Histological scoring was based on a modified semiquantitative score system described by McCafferty et al. [18]. Samples were scored in blind. Colonic changes were graded as follows: extent of mucosal architecture destruction, presence and degree of cellular infiltration, extent of muscle thickening, presence of crypt abscesses, and degree of goblet cell depletion. For each feature, a score of 0, 1, 2, or 3 was attributed, corresponding to normal, mild, moderate, and extensive changes, respectively. The scores for each feature were summed up, producing a maximum overall score of 15.

Microbiological analysis

Colon contents were removed aseptically, immediately placed into an anaerobic chamber, and dissolved in sterile pre-reduced PBS. A sterile stick was used to transfer 1 g of intestinal contents into a sterile test tube together with 2 m/ 0.9% sterile saline solution. The stool was pressed and mixed into this solution and the tube was brought to volume (10 m*l*) with 0.9% sterile saline solution. Each sample (0.1 m*l*) was serially diluted via 10-fold dilutions. Starting from the lowest concentration, dilutions were plated and cultured on different media in triplicate, using the spread plate method.

Chromocult agar and Bile Esculin Azide agar were used for the enumeration of *E. coli*/Coliforms and Enterococci, respectively. All the plates were aerobically incubated at 37°C for 24-48hr. Reinforced Clostridial agar enriched with 5% sheep blood and 1 mg/m/ vitamin K1. Brain Heart Infusion agar, Mann Rogosa Sharpe agar (MRS) and modified MRS agar, enriched with (0.3% (w/v) sodium propionate, 0.2% (w/v) lithium chloride, 0.05% (w/v) cysteine hydrochloride and 5% (v/v) defibrinated sheep blood were used for the enumeration of *Clostridium* spp*,* total anaerobes*, Lactobacillus* spp. and *Bifidobacterium* spp., respectively. Anaerobic incubation was carried out in anaerobic jars (Oxoid) at 37°C for 48-72 hr. Anaerobic conditions were obtained using Anaerogen (Oxoid) and confirmed using methyl blue strips as oxidation-reduction indicator. The number of colonies was counted and all the data are expressed as CFUxlog/g.

Statistical analysis

Diagnostic graphics were used to test assumptions and outliers. Data were analyzed using the Linear Mixed Model, where group (three levels: C, E, and TNBS), time (two levels: T0 and T1) and interaction were included as fixed factors. Sidak corrections were used for multiple comparisons. Results are expressed as estimated marginal means ± SE. Fisher's exact test was used to compare mortality rates. The Kruskal-Wallis analysis, followed by the Mann-Whitney test, was used to assess DAI and histological scores. Bacterial counts were expressed and analyzed as log10 CFU per gram of colon samples, and only the group effect was considered. The inter-group coefficient of variation (CV) was calculated as the ratio of the measurements' standard deviation divided by the mean and multiplied by 100. To evaluate the relative proportion of each examined bacteria, proportions of each bacterial group are presented where the total of the examined bacteria was set at 100%. Statistical analyses were performed using SPSS Statistics version 20 (IBM, SPSS Inc., Chicago, IL, USA). We considered $p \leq 0.05$ as significant and a p value between 0.1 and 0.05 as a trend.

Results

Mortality, Body weight, and DAI

One mouse each from the Saline and Ethanol groups died during the experimental period (11.1%; Fisher's exact test: P = 1.000). The BW reduction in the control group was not significant (P = 0.053), whereas ethanol- and TNBS-treated mice lost 9% and 10% of BW, respectively (P = 0.000). DAI was higher in the TNBS group than in the Saline group (P = 0.008; Figure 1). Ethanol-

treated mice showed no significant differences in DAI compared to the Saline and TNSB groups, probably as a consequence of the high data variability.

Morphological analysis

Colons of the saline-treated mice showed normal tissue without structural and morphological alterations (Figure 2A) Contrarily, the colon sections of mice from the Ethanol and TNSB groups showed a mild and severe grade of inflammation, respectively, characterized by infiltration of the submucosa mainly by lymphocytes and monocytes (Fig. 2B, C). The histological score was higher in the TNBS group compared to the Saline (P = 0.008) and Ethanol (P = 0.032) groups. Differences between the Ethanol and Saline groups were not significant (Figure 3)

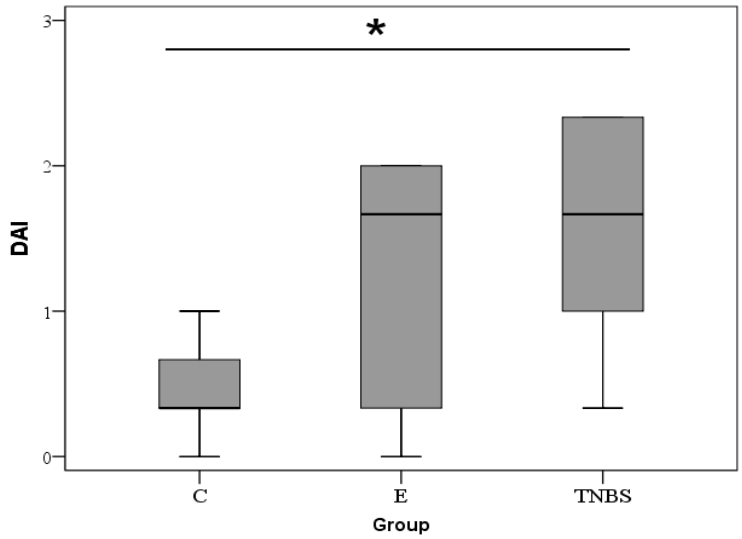

Figure 1: Disease activity index (DAI) after treatment with saline (n = 8), ethanol (n = 8) and TNBS (n = 9) in CD-1 mice determined through weight loss, stool consistency and bleeding assessment (* P < 0.05 TNBS *vs* Saline; Mann-Whitney test).

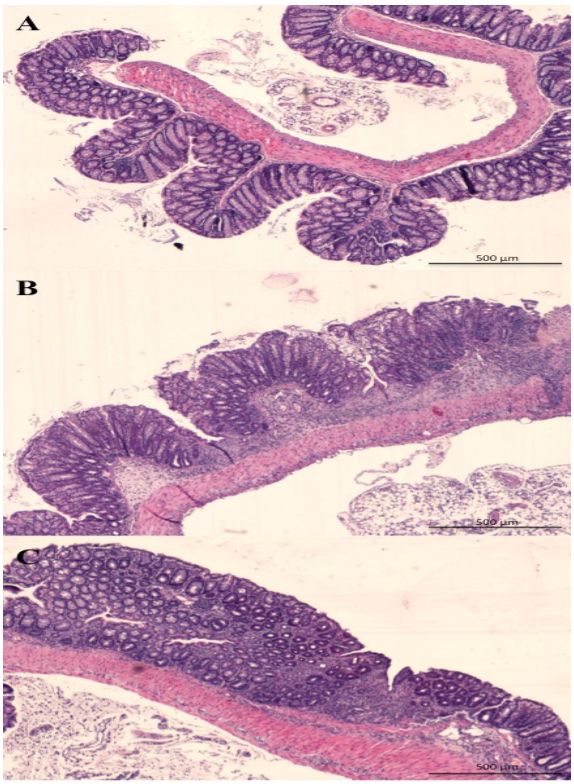

Figure 2: **A.** Colon histological section after saline solution treatment. Normal tissue without structural and morphological alterations. H&E stain, Magnification x4. **B.** Colon histological section after ethanol treatment. Mild grade of inflammation. H&E stain, Magnification x4. **C.** Colon histological section after TNBS treatment. Severe grade of inflammation. H&E stain, Magnification x4

Gut microflora

A significant effect of treatment was observed for all the bacteria examined (Figure 4): *E. coli* (P = 0.000), Enterococci (P = 0.000), Anaerobes (P = 0.000), Lactobacilli (P = 0.006), Bifidobacteria (P = 0.000), and *Clostridium* spp. (P = 0.000). *E. coli*, Enterococci, Anaerobes, and *Clostridium* spp. counts increased after both ethanol and TNBS treatments while Lactobacilli and Bifidobacteria were reduced in these groups. Inter-group CV were 15.4%, 12.0%, 5.6%, 1.4%, 23.2%, and 6.7% for *E. coli*, Enterococci, Anaerobes, Lactobacilli, Bifidobacteria, and *Clostridium* spp., respectively.

Figure 5 shows the relative proportions of bacterial species in the colon. TNBS treatment increased *E. coli* and Enterococci proportions (P = 0.000) compared to the Saline and Ethanol groups. Anaerobes were 51% and 32% higher in the Ethanol and TNBS groups, respectively, compared to saline-treated mice (P = 0.000). Conversely, the Saline group showed a higher proportion of Lactobacilli compared to the Ethanol and TNBS groups with 29% and 27% respectively (P = 0.000). Bifidobacteria proportion was dramatically reduced after both ethanol and TNBS treatments compared to saline (P = 0.000). Relative proportions of *Clostridium* spp. were small (< 0.001%) and there were no differences between groups (P = 0.119; data not shown).

Discussion

In the present study, we evaluate the effects of TNBS administration on microbiota composition, clinical signs and colon inflammatory changes in CD1 mice. TNBS was dissolved in ethanol and administered to mice in order to induce a transmural colitis, reproducing human IBD (e.g. Crohn's disease, at the histological and immunologic level). Ethanol is not used as a solvent or carrier only, as it also aids induction of gut inflammation by breaking the mucosal barrier [12]. Our data showed a reduction of BW in mice treated with ethanol. This initial weight loss is due to a nonspecific toxic destruction of the mucosa by the ethanol, as confirmed by histological examination. However, these mice showed no significant differences in terms of clinical signs, evaluated by DAI, when compared to the saline-treated animals. Moreover, the Ethanol group displayed a lower histological score than the TNBS group. Our

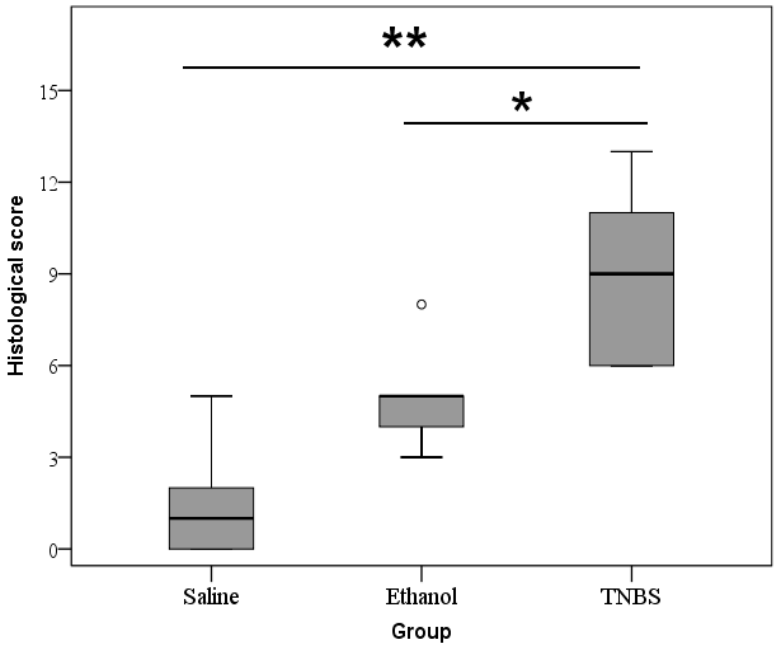

Figure 3: Histological score of different treatment groups (** P < 0.01 TNBS *vs* Saline; * P < 0.05 TNBS *vs* Ethanol; Mann-Whitney test).

findings clearly show that TNBS determines severe inflammation as demonstrated by transmural infiltration, high DAI and histological score. TNBS is a hapten agent that induces a Th1-mediated immune response involving various pro-inflammatory cytokines [12]. Nevertheless, the exact mechanisms responsible for TNBS-induced IBD are poorly understood. Various authors have proposed different mechanisms to explain the pathophysiological features of TNBS-induced IBD. It has been reported that following TNBS administration, L-type Ca2+ channels are downregulated and

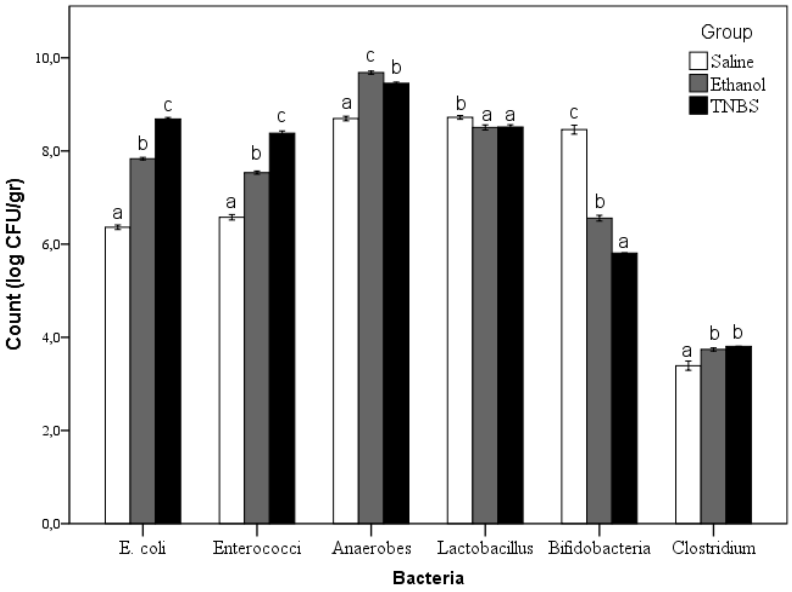

Figure 4: Effects of saline, ethanol, and TNBS treatments on colon microbiota (Log CFU/gr) in mice. Bars (mean ± SE) with no common letter within a bacterial species differ significantly ($P < 0.05$).

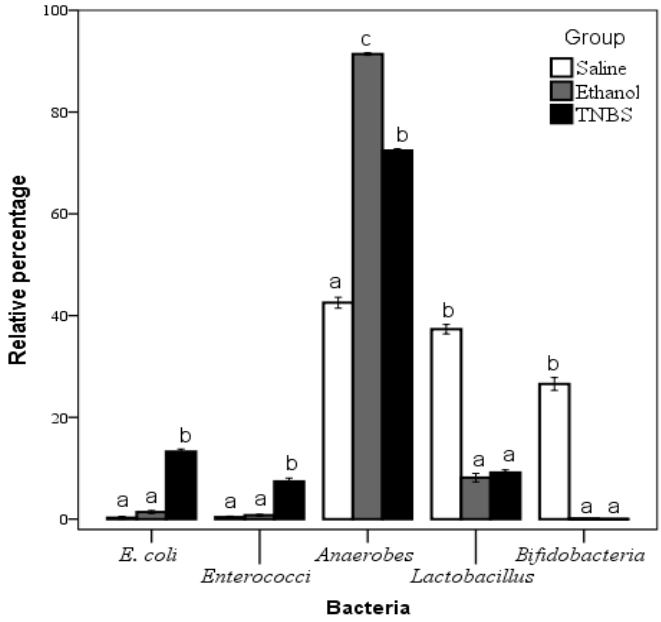

Figure 5: Effects of saline, ethanol, and TNBS treatments on colon microbiota (Log CFU/gr) in mice. Bars (mean ± SE) with no common letter within a bacterial species differ significantly ($P < 0.05$).

adenosine triphosphate (ATP)-sensitive K+ channels are upregulated in mouse gastrointestinal smooth muscle cells. These modulations induce hyperpolarization of the smooth muscle cell membrane, resulting in reduced colonic contractility [19]. A recent study reported that exposure to TNBS caused a marked decrease in both the mRNA and protein expression of aquaporin 3 and 8, suggesting that they may be involved in the pathogenesis of IBD [20].

Future studies using molecular approaches, such as that applied in a previous study [21], are needed to assess whether TNBS treatment is able to induce differential gene expression.

Disruption of the intestinal homeostasis and tolerance towards the resident microbiota is probably the key mechanism involved in the development of IBD. Indeed, the most common site of IBD is the colon, where the highest intestinal bacterial concentrations are found [1]. Over 90% of gut microbiota is composed of four major phyla. The most abundant phyla are Firmicutes (49–76%), in particular Clostridium XIV and IV groups, and Bacteroidetes (16–23%), followed by the less profuse Proteobacteria and Actinobacteria phyla [3]. Several lines of evidence suggest a key role of the gut microbiota in the pathogenesis and persistence of IBD-associated inflammation [1,22]. Different studies have shown that the gut microbiota in healthy subjects is almost temporally stable, whereas in IBD patients it is unstable with clear changes. In particular, IBD patients present an abnormal intestinal microflora (dysbiosis) in the faeces and mucosa-associated microbiota [23]. Dysbiosis is characterized by a reduction in the biodiversity of microbiota, largely due to a decline in the diversity of Firmicutes and a depletion of some bacterial phyla [24]. In particular, in IBD patients, a reduction has been observed in the relative abundance of potentially protective bacteria species, often referred to as "beneficial bacteria" (Bifidobacteria and Lactobacilli), together with a concurrent increase in potentially dangerous bacteria (i.e. *E. coli* and sulphate-reducing bacteria) [6]. However, whether the dysbiosis is the initiating factor that subsequently contributes to the development and persistence of IBD, or a consequence of impaired immunity still remains to be elucidated.

Abnormal microbiota can affect the mucosal immune system. It has been suggested that an abnormal intestinal microbiota induces an aberrant immune response of the gut immune system, resulting in a chronic inflammation of the gastro-intestinal tract in genetically predisposed subjects [25]. While some bacteria are inducers of disease, others, known as probiotics, are able to reduce the inflammatory state. In this context, recent studies have reported that supplementation with specific strains of probiotics exerts antigenotoxic, antioxidant, and anti-inflammatory effects [13,26,27].

The microbiological results of the present study indicate that mouse microbiota is susceptible to the effects of ethanol and TNBS. In particular, the bacteria more responsive to such treatments were *E. coli*, Enterococci and Bifidobacteria. Count and relative percentage of *E. coli* and Enterococci increased after TNBS treatment according to the results obtained by Ondeerdonk et al. [28], who found that luminal concentrations of these bacterial species correlated to the aggressiveness of colitis in B27 transgenic rats. Moreover, Conte et al. [29] observed an increase in aerobes and *E. coli* in pediatric ulcerative colitis. *E. coli* seems to have a role as a pro-inflammatory agent. Recent research has pointed out that the intestinal microenvironment in IBD patients would predispose to *E. coli* proliferation. Moreover, this bacterium frequently carries virulence genes related to cytotoxicity and genotoxicity, which can contribute to mucosal inflammation and tissue damage [30].

Our results showed an increase of *Clostridum* spp. counts after both ethanol and TNBS treatment. In the severe combined immune deficiency murine model of IBD, clostridia-related Gram-positive bacteria are essential for the induction of severe inflammation. We observed a higher count and relative percentage of Lactobacilli and Bifidobacteria in saline-treated

mice compared to ethanol- and TNBS-treated animals. It has been reported that a high abundance of *Lactobacillus* spp. and Bifidobacteria strongly correlate with low levels of inflammation in mice [31]. Similarly, Bullock et al. [32] observed that during active ulcerative colitis, *Lactobacillus salivarius* and *L. manihotivorans* were absent in fecal samples and reappeared with the colitis in remission. A decreased *Bifidobacterium spp.* count was also observed in subjects affected by UC and CD in a study conducted by Fyderek et al. [10]. It should be noted that the lactobacilli bacteria can inhibit intestinal inflammation as demonstrated by Petrof et al. [5], and that *Bifidobacterium spp.* participates in immune modulation and in intraepithelial lymphocyte expansion [33].

In conclusion, although dysbiosis has been linked to IBD, it is still essential to clarify whether abnormal microbiota is the initiating factor that contributes to the development and persistence of IBD, or a secondary symptom of gut inflammation. Therefore, a complete understanding of the composition and function of the gut microbiota is critical. The results of the present study demonstrate that TNBS treatment changes colonic microbiota in CD-1 mice by increasing the count and relative percentage of detrimental bacteria and by decreasing beneficial bacteria. The bacteria more responsive to treatments were *E. coli*, Enterococci and Bifidobacteria. Our study confirms that TNBS-induced colitis is a suitable model to study the pathophysiology of IBD in mice. Understanding the relationship between the microbiota and the gut immune system should lead to an enhanced understanding of the pathogenesis of IBD and to the development of curative treatments. Finally, gut microbiota composition may be useful as a diagnostic tool or biomarker for IBD once identification of the specific core microbiome will be possible.

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