Universitat Autònoma de Barcelona

Gut Commensal Microbiota and Intestinal Inflammation: Modulatory Role of Rifaximin

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Gut Commensal Microbiota and Intestinal Inflammation: Modulatory Role of Rifaximin

by

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Vicente Martínez Perea,

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I hereby declare that the Thesis entitled "*Gut Commensal Microbiota and Intestinal Inflammation: Modulatory Role of Rifaximin*", submitted by **MARINA FERRER CLOTAS** in partial fulfillment of the requirements for the degree of Doctor of Philosophy, has been carried out under my supervision and I authorize the submission to undertake its oral defense.

In witness whereof, I hereby sing this document.

Bellaterra, Barcelona, November 2019

Vicente Martínez Perea, DVM, PhD

Ph.D. Advisor

"*La perfección se alcanza, no cuando no hay nada más que añadir, sino cuando ya no queda nada más que quitar*"

Antoine de Saint-Exupéry, ESCRITOR

"*I can't change the direction of the wind, but I can adjust my sails to always reach my destination*"

Jimmy Dean, MUSICIAN & ENTREPRENEUR

Agraïments

Després de tots aquests anys em costa creure que la tesi que tinc a les mans sigui la meva. Encara que sembli que no, tot arriba. No sempre ha sigut fàcil, però ara que la veig acabada sento una satisfacció immensa. Mirant enrere m'emporto un bon record.

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Marina

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Abbreviations

16S: 16 Svedberg units

AM: ante meridiem

ANOVA: analysis of variance

AMP: antimicrobial peptide

ß-2-m: β-2-microglobulin

BW: body weight

CAS: Chemical Abstracts Service

CCL5: chemokine ligand 5

CCR5: chemokine receptor 5

CD: Crohn's disease

cDNA: complementary DNA

Ct: cycle threshold

DAMPs: damage-associated molecular patterns

DAPI: 4',6-diamidino-2-phenylindole

DC: dendritic cell

Def: defensin

DNA: deoxyribonucleic acid

DNBS: dinitrobenzene sulfonic acid

DSS: dextran sulfate sodium

FAE: follicle-associated epithelium

FDA: Food and Drug Administration

FDR: false discovery rate

FISH: fluorescent in situ hybridization

GALT: gut-associated lymphoid tissue

GCM: gut commensal microbiota

GE: genetically engineered

GI: gastrointestinal

hPXR: humanized PXR

IBD: Inflammatory bowel disease

IBS: Irritable bowel syndrome

IEC: intestinal epithelial cell

IEL: intraepithelial lymphocyte

IFN-γ: interferon-gamma

IL: interleukin

IgA: Immunoglobulin A

kD: kilodalton

LPS: lipopolysaccharide

MAMPs: microbe-associated molecular patterns

mRNA: messenger RNA

NF-κB: nuclear factor kappa beta

OTU: operational taxonomic unit

PAMPs: pathogen-associated molecular patterns

PBS: phosphate buffer solution

PCoA: principal coordinates analysis

PERMANOVA: permutational multivariate analysis of variance

PO: per os (orally)

PRR: pattern recognition receptor

PXR: pregnane X receptor

RANTES: regulated upon activation, normal T cell expressed and secreted

RegIIIγ: regenerating islet-derived protein 3 gamma

RELM-β: resistin-like molecule- beta

RNA: ribonucleic acid

rRNA: ribosomal RNA

RT-qPCR: reverse transcription quantitative polymerase chain reaction

SCFAs: short chain fatty acids

SD: standard deviation

SEM: standard error mean

SIBO: small intestine bacterial overgrowth

ssRNA: single-stranded RNA

sIgA: secretory immunoglobulin A

dsRNA: double-stranded RNA

TFFs: trefoil factor peptides

TGFβ: transforming growth factor-beta

TLR: Toll-like receptor

TNBS: trinitrobenzene sulfonic acid

TNFα: tumor necrosis factor alpha

tRF: theoretical restriction 5'-fragment

T-RFLP: terminal restriction fragment length polymorphism

UC: ulcerative colitis

uc: unclassified

v:v:v: volume/volume/volume

wt/v: weight/volume

Summary

Inflammation of the gastrointestinal tract is a common component of functional gastrointestinal disorders (irritable bowel syndrome, IBS and inflammatory bowel disease, IBD). Evidences indicate that both arise because of a convergence of altered microbiota and external environmental factors in genetically susceptible individuals, leading to abnormal immune responses and the development of persistent inflammation, through a mechanism not fully understood. Given the important role of the microbiota and the immune system in their pathogenesis, immunomodulatory antibiotics are an interesting therapeutic approach, targeting simultaneously the microbiota and the exacerbated inflammatory response. Rifaximin is a non-absorbable antibiotic approved for the treatment of IBS with diarrhea and with beneficial effects in IBD. However, the mechanisms mediating these effects and the exact contribution of its antimicrobial and immunomodulatory activities are not fully understood.

This work explores the mechanisms of action of rifaximin modulating gut microbiota and the local immune system in a healthy state and during acute intestinal inflammation using a dextran sulfate sodium (DSS)-induced colitis mouse model. First, healthy female mice were treated with either vehicle or rifaximin during 7 or 14 days. In a second study, colitis was induced with DSS (3% in water, 5 days). Mice were treated, in a preventive manner, with either rifaximin or doxycycline, serving as positive control. Daily clinical signs were recorded. At necropsy, colonic inflammation was assessed (macroscopic signs and histopathology). Luminal and wall-adhered ceco-colonic microbiota were characterized by fluorescent *in situ* hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA gene sequencing. Local immune responses and host-bacterial interactions were determined assessing the expression (RT-qPCR) of pro- and anti-inflammatory cytokines, antimicrobial peptides and Toll-like receptors (TLRs).

In healthy mice, rifaximin did not modify neither total ceco-colonic bacterial counts not microbial biodiversity. Moreover, rifaximin was associated to a minor upregulation of TLRs expression, without changes in the expression of immune-related markers. Animals receiving DSS showed clinical signs indicative of the development of colitis. Rifaximin did not affect the clinical course of colitis, while doxycycline attenuated clinical signs. Similarly, colitis-associated up-regulation of immune-related markers was not affected by rifaximin, while doxycycline completely prevented this response. As it relates to host-bacterial interaction markers, colitis selectively up-regulated the antimicrobial peptide RegIIIɣ, while it had minor effects on TLRs expression. Rifaximin did not affect colitis-associated RegIIIɣ up-regulation, while doxycycline completely normalized its expression. The pregnane X receptor (PXR) was down-regulated during colitis, a change not affected by rifaximin but prevented by doxycycline. DSS-induced colitis was associated to a dysbiotic state characterized by an increase in Verrucomicrobia and Deferribacteres and a simultaneous decrease in Bacteroidetes; with a maintenance of alpha diversity. During rifaximin treatment, with or without colitis, bacterial richness was maintained, with a bacterial composition closely related to that observed in healthy animals, vs. that observed during colitis. Doxycycline-treated animals showed extensive changes in their microbiota, with similarities between the inflamed and non-inflamed conditions. Particularly, doxycycline reduced pathogenic Clostridiales, while increased Clostridia cluster XIVa-related, *Lactobacillus* and *Ruminococcus*, considered beneficial groups.

These results show that in healthy animals rifaximin has limited antimicrobial/immunomodulatory activities. Moreover, in the DSS-induced colitis model, rifaximin does not exhibit significant immunomodulatory nor microbial-modulatory effects consistent with an anti-inflammatory activity. These negative findings do not exclude an immunomodulatory and/or microbial-modulatory action of rifaximin in humans, thus explaining its effectiveness in IBS and IBD. Taking into account the, at least partially, postulated mechanism of action of rifaximin through human PXR receptors, the DSS-induced colitis model in mice might not be adequate to fully address the mechanisms of action of rifaximin modulating intestinal inflammation. Doxycycline showed positive anti-inflammatory effects and induced an apparent protective dysbiotic state, thus warranting further studies.

Resumen

Los trastornos funcionales gastrointestinales (síndrome del intestino irritable) y la enfermedad inflamatoria intestinal presentan una inflamación crónica del tracto gastrointestinal. Aunque su patogenia no se conoce totalmente, ambas enfermedades resultan de la interacción de la microbiota con factores ambientales que convergen en individuos genéticamente susceptibles, dando lugar a una activación anómala del sistema inmune. Los antibióticos con actividad inmunomoduladora son una opción terapéutica interesante por su acción simultánea frente la microbiota y la reacción inmunitaria. La rifaximina es un antibiótico no absorbible aprobado para el tratamiento del síndrome del intestino irritable con diarrea y con efectos beneficiosos en la enfermedad inflamatoria intestinal. Sin embargo, se desconoce su mecanismo de acción y la contribución de sus efectos antimicrobianos e inmunomoduladores.

Este trabajo explora el mecanismo de acción de la rifaximina en ratones sanos y durante la inflamación intestinal aguda. Se valoraron los efectos de la rifaximina en animales sanos y en animales con colitis aguda, utilizando el modelo de colitis inducida por dextrano sulfato sódico (DSS, 3% en agua, 5 días). Los ratones se trataron preventivamente con rifaximina o doxiciclina (control positivo). Se valoraron los signos clínicos de inflamación y el estado del colon (macroscópicamente e histopatología) en el momento de la necropsia. La microbiota ceco-cólica, luminal y adherida al epitelio, se caracterizó mediante: hibridación *in situ* fluorescente (FISH), polimorfismos de longitud de fragmentos de restricción (T-RFLP) y secuenciación del 16S ARNr. La respuesta inmune local y los mecanismos de interacción hospedador-microbiota se valoraron determinando cambios en la expresión génica (RT-qPCR) de citoquinas pro- y anti-inflamatorias, péptidos antimicrobianos y receptores de tipo Toll (TLRs).

En animales sanos, la rifaximina no modificó los recuentos totales de bacterias ceco-cólicas, su biodiversidad o la adherencia bacteriana a la pared del colon. Sólo se observaron cambios menores en la expresión de TLRs o de otros marcadores de tipo inmune. Los animales que recibieron DSS mostraron signos clínicos indicativos del desarrollo de colitis, con regulación al alza en la expresión de marcadores inmunes, incluyendo al péptido antimicrobiano RegIIIγ. La rifaximina no afectó el curso clínico de la colitis ni la expresión de dichos marcadores. Por el contrario, la doxiciclina atenuó ambas respuestas. Con independencia del tratamiento aplicado, sólo se observaron cambios menores en la expresión de TLRs. La expresión del receptor X de pregnano (PXR) disminuyó durante la inducción de la colitis, un cambio no modificado por la rifaximina, pero que se previno con la doxiciclina. La colitis indujo una disbiosis caracterizada por un aumento de Verrucomicrobia y Deferribacteres con un descenso de Bacteroidetes, manteniéndose la diversidad de tipo alfa. Durante el tratamiento con rifaximina, con o sin colitis, se mantuvo la biodiversidad, con una composición bacteriana muy similar a la observada en animales sanos. Los animales tratados con doxiciclina mostraron cambios extensos en la microbiota, con similitudes entre los grupos inflamado y no inflamado. Concretamente, la doxiciclina redujo los grupos Clostridiales patogénicos, mientras que aumentó los Clostridia grupo XIVa, *Lactobacillus* and *Ruminococcus*, considerados grupos beneficiosos.

Estos resultados muestran que en animales sanos la rifaximina presenta una actividad antimicrobiana e inmunomoduladora limitada. En estado de colitis, la rifaximina no manifiesta efectos inmunomoduladores ni antimicrobianos consistentes con una actividad anti-inflamatoria. Sin embargo, estas acciones no pueden excluirse en humanos, explicando los efectos beneficiosos observados clínicamente. Teniendo en cuenta que en humanos la rifaximina actuaría a través de los PXR, es posible que el modelo de colitis inducida por DSS en ratón no sea el adecuado para estudiar su mecanismo de acción. La doxiciclina mostró efectos anti-inflamatorios e indujo un estado de disbiosis aparentemente protector frente a la inflamación, aunque se requieren más estudios para confirmarlo.

Resum

Els trastorns funcionals gastrointestinals (síndrome de l'intestí irritable) i la malaltia inflamatòria intestinal presenten una inflamació crònica del tracte gastrointestinal. Encara que la seva patogènia no es coneix totalment, totes dues malalties resulten de la interacció de la microbiota amb factors ambientals que convergeixen en individus genèticament susceptibles, donant lloc a una activació anòmala del sistema immune. Els antibiòtics amb activitat immunomoduladora són una opció terapèutica interessant per la seva acció simultània davant la microbiota i la reacció immunitària. La rifaximina és un antibiòtic no absorbible aprovat pel tractament de la síndrome de l'intestí irritable amb diarrea i amb efectes beneficiosos en la malaltia inflamatòria intestinal. No obstant, es desconeix el seu mecanisme d'acció i la contribució dels seus efectes antimicrobians i immunomoduladors.

Aquest treball explora el mecanisme d'acció de la rifaximina en ratolins sans i durant la inflamació intestinal aguda. Es valoraren els efectes de la rifaximina en animals sans i en animals amb colitis aguda, utilitzant el model de colitis induïda per dextrà sulfat sòdic (DSS, 3% en aigua, 5 dies). Els ratolins es tractaren preventivament amb rifaximina o doxiciclina (control positiu). Es valoraren els signes clínics d'inflamació del còlon (macroscòpicament i histopatologia) en la necròpsia. La microbiota ceco-còlica, luminal i adherida a l'epiteli, es caracteritzarà mitjançant: hibridació in situ fluorescent (FISH), polimorfismes de longitud de fragments de restricció (T-RFLP) i seqüenciació del 16S ARNr. La resposta immune local i els mecanismes de interacció hoste-microbiota es valoraren determinant canvis en l'expressió gènica (RT-qPCR) de citocines pro- i antiinflamatòries, pèptids antimicrobians i receptors de tipus Toll (TLRs).

En animals sans, la rifaximina no va modificar els recomptes totals, la biodiversitat o la adherència a la paret del còlon dels bacteris ceco-còlics. Només es van observar canvis menors en l'expressió de TLRs o d'altres marcadors de tipus immune. Els animals que van rebre DSS van mostrar signes clínics indicatius del desenvolupament de colitis, amb regulació a l'alça en l'expressió de marcadors immunes, incloent el pèptid antimicrobià RegIIIγ. La rifaximina no va afectar el curs clínic de la colitis ni l'expressió d'aquests marcadors. Per contra, la doxiciclina va atenuar les dues respostes. Independentment del tractament aplicat, només es van observar canvis menors en l'expressió de TLRs. L'expressió del receptor X de pregnà (PXR) va disminuir durant la inducció de la colitis, un canvi no modificat per la rifaximina, però que es va prevenir amb la doxiciclina. La colitis va induir una disbiosi caracteritzada per un augment de Verrucomicrobia i Deferribacteres amb un descens de Bacteroidetes, mantenint-se la diversitat de tipus alfa. Durant el tractament amb rifaximina, amb o sense colitis, es va mantenir la biodiversitat, amb una composició bacteriana molt similar a l'observada en animals sans. Els animals tractats amb doxiciclina van mostrar canvis extensos en la microbiota, amb similituds entre els grups inflamat i no inflamat. Concretament, la doxiciclina va reduir els grups Clostridiales patogènics, mentre que va augmentar els Clostridia grup XIVa, *Lactobacillus* i *Ruminococcus*, considerats grups beneficiosos.

Aquests resultats mostren que en animals sans la rifaximina presenta una activitat antimicrobiana i immunomoduladora limitada. En estat de colitis, la rifaximina no manifesta efectes immunomoduladors ni antimicrobians consistents amb una activitat antiinflamatòria. No obstant això, aquestes accions no es poden excloure en humans, explicant els efectes beneficiosos observats clínicament. Tenint en compte que en humans la rifaximina actuaria a través dels PXR, és possible que el model de colitis induïda per DSS en ratolí no sigui l'adequat per estudiar el seu mecanisme d'acció. La doxiciclina va mostrar efectes antiinflamatoris i va induir un estat de disbiosi aparentment protector enfront de la inflamació, encara que es requereixen més estudis per confirmar-ho.

Introduction

1 The gastrointestinal tract

The gastrointestinal (GI) tract is a continuous semipermeable tube that goes from the mouth to the anus. Its primary function is to serve as a portal through which nutrients and water can be absorbed. The wall of the GI tract is described according to its component layers, which are joined together by connective tissue and by neural and vascular elements.[1] Four layers form all segments of the GI tract: the mucosa, the submucosa, the tunica muscularis and the serosa (Fig. 1).[1–3]

- Mucosa

  The mucosa includes the lining epithelium, as well as the lamina propria and the muscularis mucosa (Fig. 1). The epithelium consists of a single layer of columnar cells, the intestinal epithelial cells (IECs). Intestinal epithelial cells are polarized cells with an apical surface facing the intestinal lumen and a basolateral surface facing the lamina propria.[4] The collagen-rich lamina propria lies underneath the epithelium and acts as a physical support to the IECs. The muscularis mucosa is a thin layer of smooth muscle that separates the mucosa from the submucosa. Throughout the small intestine, the epithelium is folded up into villi, with the infolding areas forming crypts, to maximize the surface area available for nutrient absorption. This structure is modified at the level of the large intestine, where villi are poorly developed but the structure of the crypts is maintained.

- Submucosa

  The submucosa comprises a dense collagenous stroma and contains large blood vessels, lymphatic vessels, and nerves (Fig. 1).

- Tunica muscularis

  The tunica muscularis is composed of two distinct layers of smooth muscle: an inner circular layer and an outer longitudinal layer (Fig.1).

- Serosa

  Finally, the serosa or mesothelium is a thin membrane of connective tissue formed by a layer of flattened, specialized mesothelial cells (Fig. 1).

The intestine presents different physiological functions along its length. The small intestine extends from the pylorus to the cecum and it is divided by three portions: the duodenum, the jejunum and the ileum. Its main function is the chemical digestion and the absorption of the nutrients present in the ingested food. The large intestine connects the ileum with the anus and is composed by the cecum, the colon and the rectum. The general and main functions of the large intestine are the resorption of water and the excretion of the non-digestible components of the aliments.[3]

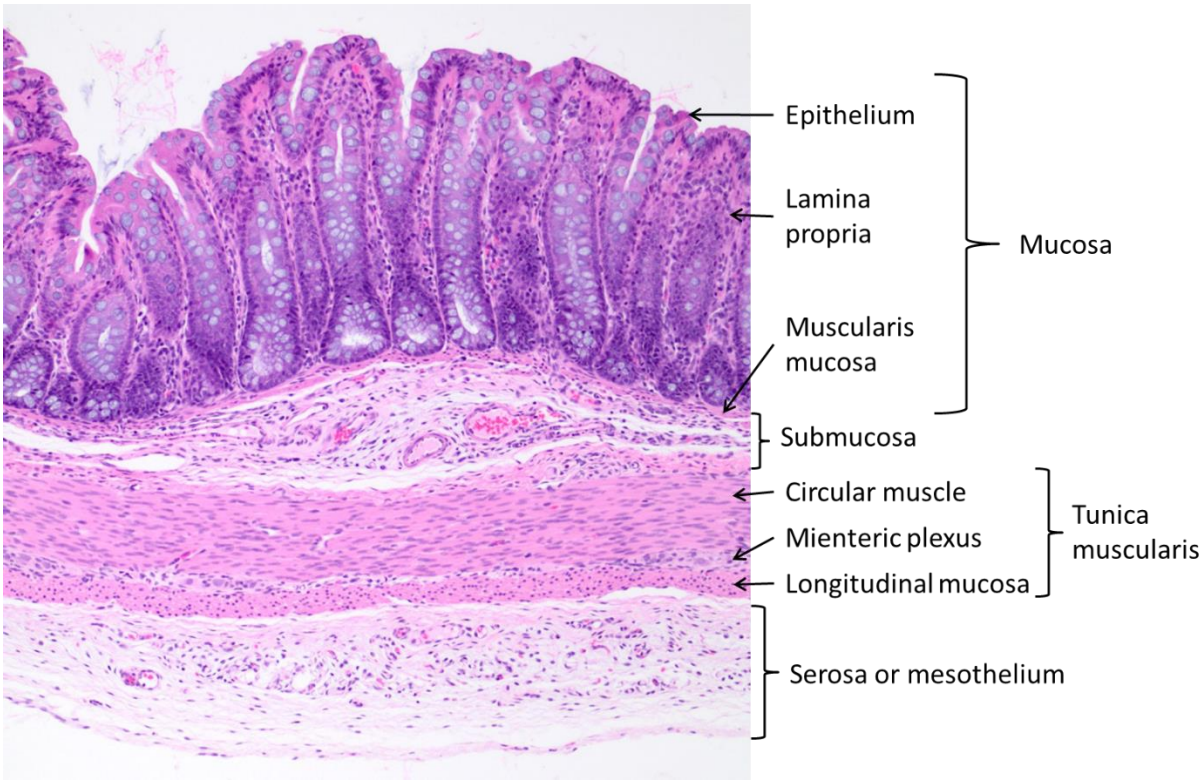

Figure 1. Histological structure of the colon. The colonic wall, as well as the rest of the gastrointestinal tract, is divided into four distinct layers: mucosa, submucosa, tunica muscularis, and serosa (see text for details). Adapted from: Jennings and Premanandan, 2017.[2]

2 Intestinal barrier

The intestinal mucosa is continuously exposed to an immense load of antigens from ingested food, resident bacteria, invading viruses, etc. Therefore, an important function of this layer is to act as a physical barrier between the external (luminal) and internal environments. In this respect, and from a functional point of view, two main barrier components are recognized throughout the GI tract: a superficial physical barrier able to avoid bacterial adhesion and to modulate paracellular diffusion to the underlying host tissues, and a deeper functional barrier, which can discriminate commensal bacteria from pathogens. Consequently, this deeper barrier is in charge of the immunological tolerance to commensals and the generation of immune responses to pathogens.[5–7]

From a histological point of view, the first layer facing the intestinal lumen is made up of IECs. IECs are closely joined together by tight junctions forming a physical barrier against the external environment. Intestinal permeability is defined as a functional feature of this barrier allowing the coexistence with bacterial symbionts necessary for our organism while preventing luminal penetration of macromolecules and pathogens.[8–10] This barrier consists of a single layer of four different specialized subtypes of cells (Fig. 2):

- Columnar absorptive enterocytes: These cells make up most of the IECs. They participate in the chemical digestion and absorption of nutrients in the small intestine and water absorption in the colon. To maximize the surface area, the apical (luminal) surface is covered with closely packed microvilli. Colonic enterocytes (colonocytes) are less active and show less microvilli than those in the small intestine. Enterocytes are also important as sensors of the microbial environment by directly interacting with the microbiota that arrives through the mucus layer.

- Paneth cells: these cells reside in the base of the crypt where they synthetize and secrete a series of antimicrobial compounds important for immunity and host-defense. In normal conditions, they are found mainly in the small intestine, but in states of inflammation they appear as a significant cellular component in the colonic mucosa.

- Mucus-producing goblet cells: these cells are specialized in the secretion of mucus, which covers the surface of the intestinal epithelium. The mucus is composed of proteins (mucins),

carbohydrates, lipids, and a high degree of water. Goblet cells also secrete a range of bioactive molecules that participate in defensive mechanisms, particularly against the microbiota.

- Hormone-producing enteroendocrine cells: these cells are part of the enteroendocrine system. They can be found throughout the entire intestine and secrete a large variety of GI regulatory peptides.

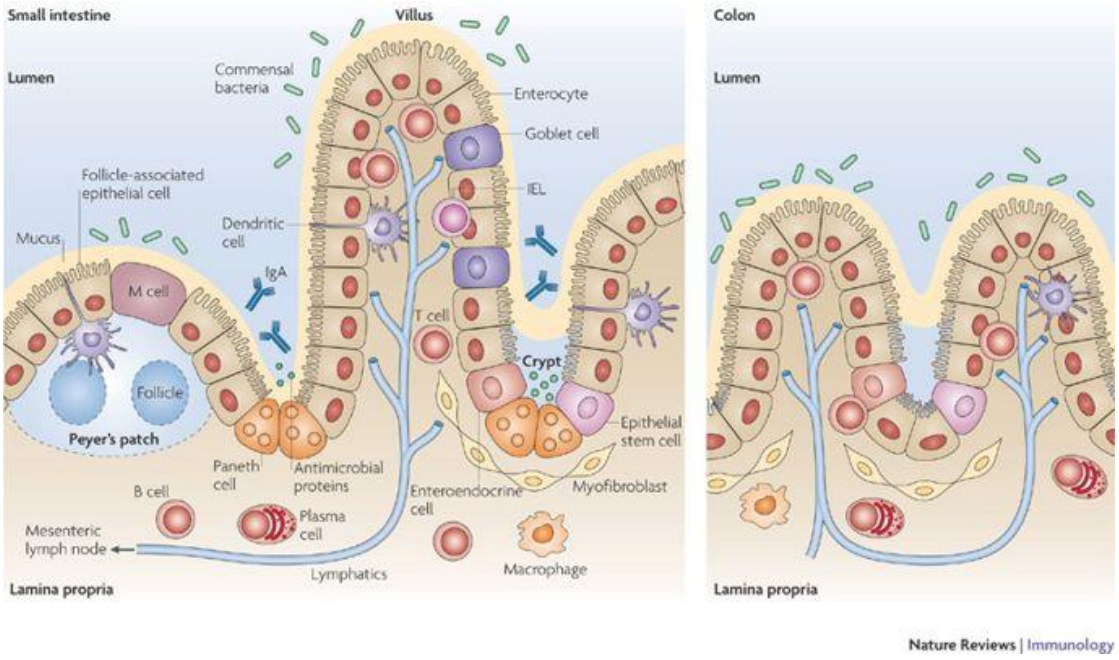

Figure 2. Anatomy of the intestinal barrier. The single layer of IECs is composed of four different specialized cells: enterocytes, goblet cells, Paneth cells and enteroendocrine cells (not represented). IECs are covered with mucus and together they form a physical barrier from the luminal contents. Stem cells are located at the base of the crypts. Underneath the IECs, the lamina propria contains specialized immune cells. Similar organization is observed in the small intestine and the large intestine. Image obtained from Abreu, 2010.[4]

All these cell types have a rather limited survival time (for instance 3 to 4 days for enterocytes). Therefore, a continuous cellular replacement is required. For this, long-lived pluripotent stem cells progenitors, located at the base of intestinal crypts, continuously produce tissue-specific precursor cells that transit through a differentiation pathway that gives rise to the different subtypes of cells present at the epithelium, as described above.[4,8,9,11]

Underneath the IECs layer, the intestinal mucosa contains a specialized immune system, the so called gut-associated lymphoid tissue (GALT). It consists of specialized immune centers that can be found along the gastrointestinal tract, like the Peyer's patches, the lymphoid follicles and the lymph nodes. This resident intestinal immune system samples the lumen to discriminate between pathogenic and harmless antigens.[12]

3 Gut commensal microbiota: From humans to mice

The intestine is an unusual organ that becomes colonized from birth with a large number of commensal bacteria, particularly in the colon. As a result, a range from 3 x 10^{13} to 40 x 10^{13} bacteria inhabits the intestinal lumen in a beneficial relationship with the host.[13,14] Throughout life, the constant interaction between external stimuli and the host at mucosal surfaces of the body is a delicate balance and the gut commensal microbiota (GCM) is a key player in maintaining homeostasis.[15]

The microbiota is dominated by bacteria, and the term is often used to define only the bacterial population. However, the term microbiota includes the entire population of microorganisms that colonizes a particular location, and includes not just bacteria, but also other microorganisms such as fungi, archaea, viruses, and protozoans. Although the term microbiota and microbiome are frequently used as synonyms, they have to be differentiated. Microbiome is a wider term since it refers to microorganisms, their collective genomes and environmental factors of a particular habitat.[16,17] Taking into account these considerations, through this work, the terms microbiota and GCM are used to refer to the bacterial population.

Overall, GCM are distributed over more than 1000 species, most of which cannot be cultured. The human gut microbiota shows a huge phylogenetic diversity in its composition. Nevertheless, it is dominated by four major phyla: Firmicutes (49 – 76%) and Bacteroidetes (16-23%) followed by Proteobacteria and Actinobacteria;[7] although Verrucomicrobia and Archaea (Euryarchaeota) are also relatively abundant.[18] The less prevalent bacterial groups are distributed among Fusobacteria and Cyanobacteria, present in very low proportions.[19] In addition to the enormous population of bacteria, the human gut has also been estimated to contain more than 10^{15} viruses and bacteriophages, as well as substantial numbers of archaea and fungi. The

complexity of the human gut microbiota is further evidenced by the spatial distribution and alternation of microorganisms throughout the length of the gastrointestinal tract (Fig. 3). The more proximal portions of the GI tract (stomach and duodenum) contain much higher levels of luminal oxygen and are populated by a higher percentage of aerobic and facultative anaerobic bacteria, although total numbers of bacteria in these regions are much lower than those found in the distal portions of the small and large intestine. The distal portions of the small intestine and the colon contain little or no oxygen, thus favoring the growth of obligate anaerobes[14] (Fig.3). Indeed, the majority of the gut microbiota is composed of strict anaerobes that populate the distal regions of the gut. *Bacteroides, Eubacterium, Bifidobacterium, Fusobacterium* and *Peptostreptococcus*, as anaerobic bacteria, predominate in the colon, whereas aerobic bacterial species including *Escherichia coli, Lactobacillus* and *Enterobacter* are less abundant. On the small intestine, Proteobacteria and Lactobacillales are dominant.[20,21]

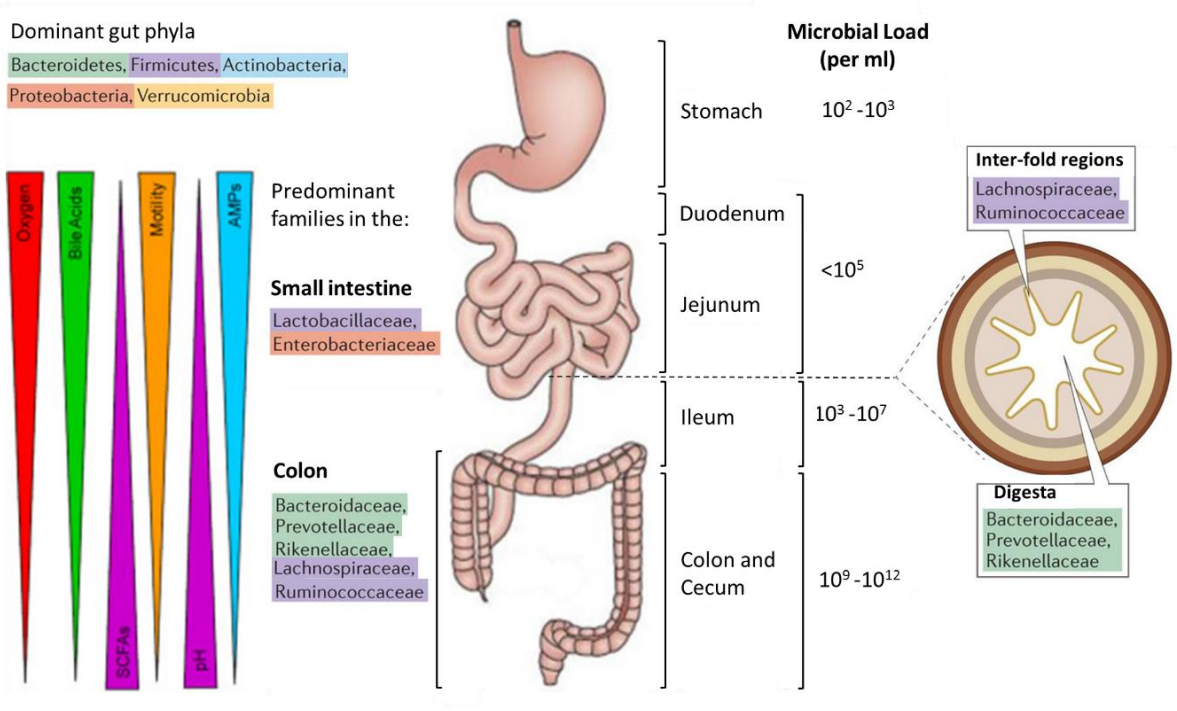

Figure. 3. Distribution of the normal GCM and physiological gradients throughout the GI tract. The proximal portions of the GI tract present higher oxygen and bile acids concentration which allows the existence of aerobic and facultative anaerobic bacteria. The hypoxic environment of the distal small intestine and colon allows the growth of obligate anaerobic bacteria. In turn, these bacteria produce high quantities of short chain fatty acids used in colonic processes. SCFAs, short chain fatty acids; AMPs, antimicrobial peptides. Adapted from Donaldson *et al.,* 2016 and Reinoso *et al.*, 2016.[14,22]

It has been demonstrated that different bacteria are also found in different layers of the gut, such as the central lumen, associated with mucosal folds, or embedded in the mucus layer. Each intestinal niche is thought to shelter the microbes that would be the most convenient to preserve local tissue homeostasis.[23]

One important finding in the inter-individual diversity in human gut microbiota was the observation of the existence of three robust clusters, designated as enterotypes. Each of the three enterotypes is identifiable by the relative abundance of one of three main bacterial genera: *Bacteriodetes* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3).[24]

Although most of the detailed characterization available of the intestinal microbiota has been performed in humans, rodent, particularly mice, GCM has been relatively well characterized. Bacterial composition can be modulated by many factors, such as the housing environment, age, sex, genetic background or diet, among others.[25–27] Therefore, the published literature contains a large variability when addressing the characterization of the microbiota in rodents. Although mice and humans share two major phyla, Bacteroidetes and Firmicutes, and approximately 80 different genera, major differences exist between these two species.[14,28,29] Two enterotypes have been identified in the laboratory mouse gut microbiota, with noticeable parallel with the human ones: enterotype 1 is dominated by *Ruminococcus* and enterotype 2 by *Bacteroides* (Fig. 4).[30] However, it is described that around 85% of bacterial groups found in the mouse gut microbiota are not present in human. Moreover, the relative abundances of most dominant genera are somewhat different between the two species. However, it is worth to note that most human gut microbiome studies use stool samples, whereas cecal and ceco-colonic contents are usually used in mouse studies, with significant differences in microbial composition described for fecal vs. cecal/ceco-colonic samples.[28] Bacterial groups found in human gut microbiota in relatively high abundance include *Prevotella*, *Faecalibacterium* and *Ruminococcus*, whereas *Lactobacillus*, *Alistipes* and *Turicibacter* are the more frequently described in mouse gut microbiota. *Clostridium, Bacteroides* and *Blautia*, on the contrary, have a similar relative abundance in both species. Moreover, *Faecalibacterium* is reported to be very important in healthy gut microbiota in humans although it is not detected, or has a very low relative abundance, in mice.[28] However, all these differences should be interpreted cautiously due to the difficulty of translating the results of mouse studies to human. Moreover, an organism cannot be reported as absent based on its lack in community studies. Thus, although absolute comparisons are difficult, murine models are helpful for studying basal microbiota and shifts upon disturbance.[28]

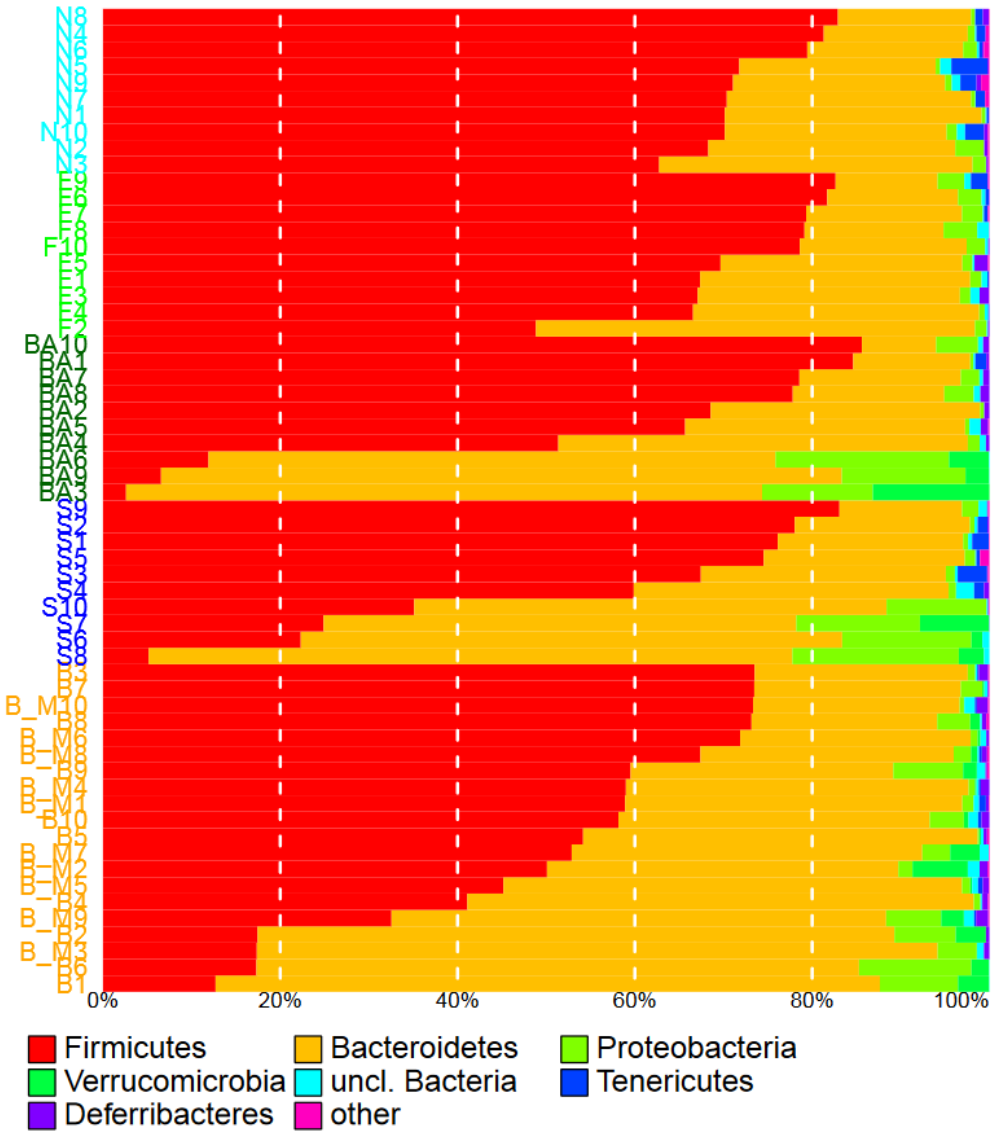

Figure 4. Overview of GCM in mice at the phylum level. The image shows relative abundance of the GCM, at the phylum level, in different mice strains (N: NOD, F: FVB, BA Balb/c; S: Swiss; B: B6). The existence of two basic enterotypes can be appreciated: enterotype 1, dominated by *Ruminococcus* (phylum Firmicutes) and enterotype 2, dominated by *Bacteroides* (phylum Bacteroidetes). From Hildebrand *et al.*, 2013.[30]

Gut microbiota plays a key role in food digestion, drug metabolism, detoxification, vitamin production, and prevention of pathogenic bacteria adhesion.[31,32] Moreover, the microbiota plays an essential role in the function, induction, and training of the host immune system.[18] During the last years, there has been a growing interest in GCM and its interactions with the host. Host-bacterial interactions can be defined as a balance between symbiosis, commensalism, and pathogenicity.[33] The beneficial relationship between the host and the gut bacteria is called

symbiosis and assumes a balance in the microbiota; on the other hand a shift in the balance of the gut microbiota is referred as dysbiosis and can entail alterations in host-bacterial interactions.[7]

3.1 Techniques for the characterization of gut microbiota

A good characterization of the microbiota is key to properly recognize states of dysbiosis and to understand the relationship between microbiota and disease.[34] The GCM contains a large proportion of unculturable bacteria, thus difficult to characterize using classical microbiological methods.[35,36] Therefore, the development of culture-independent techniques have revolutionized our knowledge of the gut microbiota. Overall, these techniques are based on the characterization and analysis of sequence divergences of the bacterial small subunit ribosomal RNA (16S rRNA). Based on this principle, widely used techniques for the study of the microbiota include terminal restriction fragment length polymorphism (T-RFLP), fluorescence *in situ* hybridization (FISH) and next-generation sequencing of the 16S rRNA gene or its amplicons (metagenomics).[34]

- Terminal restriction fragment length polymorphism: T-RFLP is a molecular biology technique consisting in the amplification and subsequent digestion, by one or more restriction enzymes, of the 16S rRNA genes present in a community sample. Thereafter, fragments are separated according to their length by gel electrophoresis allowing the detection of different size bands. Each band corresponds to a specific sequence and potentially represents a separate bacterial species, generating a fingerprint of an unknown microbial community.[34]

- Fluorescence *in situ* hybridization: FISH is a molecular technique based on the use of specific oligonucleotide probes targeting the 16S ribosomal RNA, being useful for the detection of both culturable and unculturable bacteria.[37–40] The hybridization of specific regions within the bacterial ribosome has been proven to be a useful tool for bacterial quantification and identification at the genus level.[25,26,41,42] Applied to histological samples, this technique has been used to assess the spatial location of bacteria within the intestinal wall, in particular epithelial attachment and mucus distribution.[26,41]

- Metagenomics: Metagenomics is the current gold standard for taxonomic identification of gut microbiota. The two main next generation sequencing techniques used are: 16S rRNA gene sequencing, based on the sequencing of the 16S ribosomal gene; and whole metagenomics shotgun sequencing, a more recent method based on the sequencing of all the genes in all microorganisms present in a sample.[43,44] 16S rRNA gene sequencing requires intense bioinformatical analysis to classify representative sequences obtained to the corresponding organisms that have been cataloged in known reference databases.[34,45] Taxonomic classification of samples sequenced through this method tends to be constrained to the phylum, family and genus taxonomic levels. Nevertheless, additional subsequent non-taxonomic analysis can be applied. In this analysis, clones with more than 97% sequence similarity are grouped into the so-called Operational Taxonomic Units (OTUs)[46] (in some cases also called "molecular species"[13]). Thus, from a practical point of view, OTUs are similar to the species taxonomic level.[46,47] Each OTU is named according to the first clone identified that had the representative sequence analyzed. Therefore, OTUs are generated as an intra-experiment approximation of bacterial species.[46,47]

Biodiversity and richness are an important feature of a healthy microbiota. Overall microbiota diversity for a given pool of samples is described in terms of alpha and beta diversity.

- Alpha diversity: Alpha diversity is a measure of within samples diversity and provides a relative quantification of the microbial diversity present in a given sample after its metagenomic analysis. Commonly used indices to estimate alpha diversity include the Shannon diversity index[48] and the Sobs index (species richness). Sometimes, the absolute number of OTUs identified in a sample is also given as a measure of alpha diversity.

- Beta diversity: Beta diversity is a measure of between samples diversity when comparing different populations (different environments) and serves as a measure of differences in taxonomic abundance profiles. Beta diversity indices are usually descriptive or semi-quantitative. The more used ones are phylogenetic trees and the Bray-Curtis dissimilarity index. In particular, the Curtis dissimilarity index is based on a principal coordinates analysis (PCoA), allowing a visual representation of the microbial compositional differences among samples by reducing samples variables in a two- or three-dimensional scatterplot[49] and the subsequent grouping of samples in clusters of similarity.

The massive quantities of data resulting from these technologies have vastly improved the ability to characterize microbial communities. However, the complex methodologies are accompanied by high variability that has to be optimized to allow feasible cross-studies reproducibility and comparability.[50] Moreover, using metagenomics alone does not complete the study the whole microbiome lacking information related to changes in gene expression and metabolic byproducts.[44]

4 Host-bacterial interactions

The gut microbiota plays an essential role in the maintenance of intestinal homeostasis and the development of a fully functional immune system.[14] This healthy coexistence is maintained by a variety of immune-related mechanisms that include the recognition and toleration of commensal microbiota and the generation of defensive responses against pathogenic bacteria. These responses depend on the interaction between the host and the microbiota (the so-called host-bacterial interactions) and involve the innate and the adaptive immune systems. Both, beneficial or detrimental responses can result from host-bacterial interactions (Fig. 5). Interaction with beneficial bacteria might promote anti-inflammatory effects and the maintenance/restoration of eubiotic states. On the other hand, alterations in host-bacterial interactions, including the interaction with non-beneficial microbiota, may lead to abnormal responses, including abnormal immune responses (i.e. persistent immune activation), the development of inflammation and the generation/maintenance of states of dysbiosis (Fig. 5).
Here we will focus on two of the mechanisms of host-bacterial interactions present in the GI tract: Toll-like receptors and antimicrobial peptides.

4.1 Pattern recognition receptors: Toll-like receptors

IECs and innate immune cells of the lamina propria are able to differentiate self from non-self through the cellular expression of pattern recognition receptors (PRRs). PRRs sense microorganisms through the detection of conserved microbe-specific molecules classified as microbe-associated molecular patterns (MAMPs) or as pathogen-associated molecular patterns (PAMPs).[51,52]

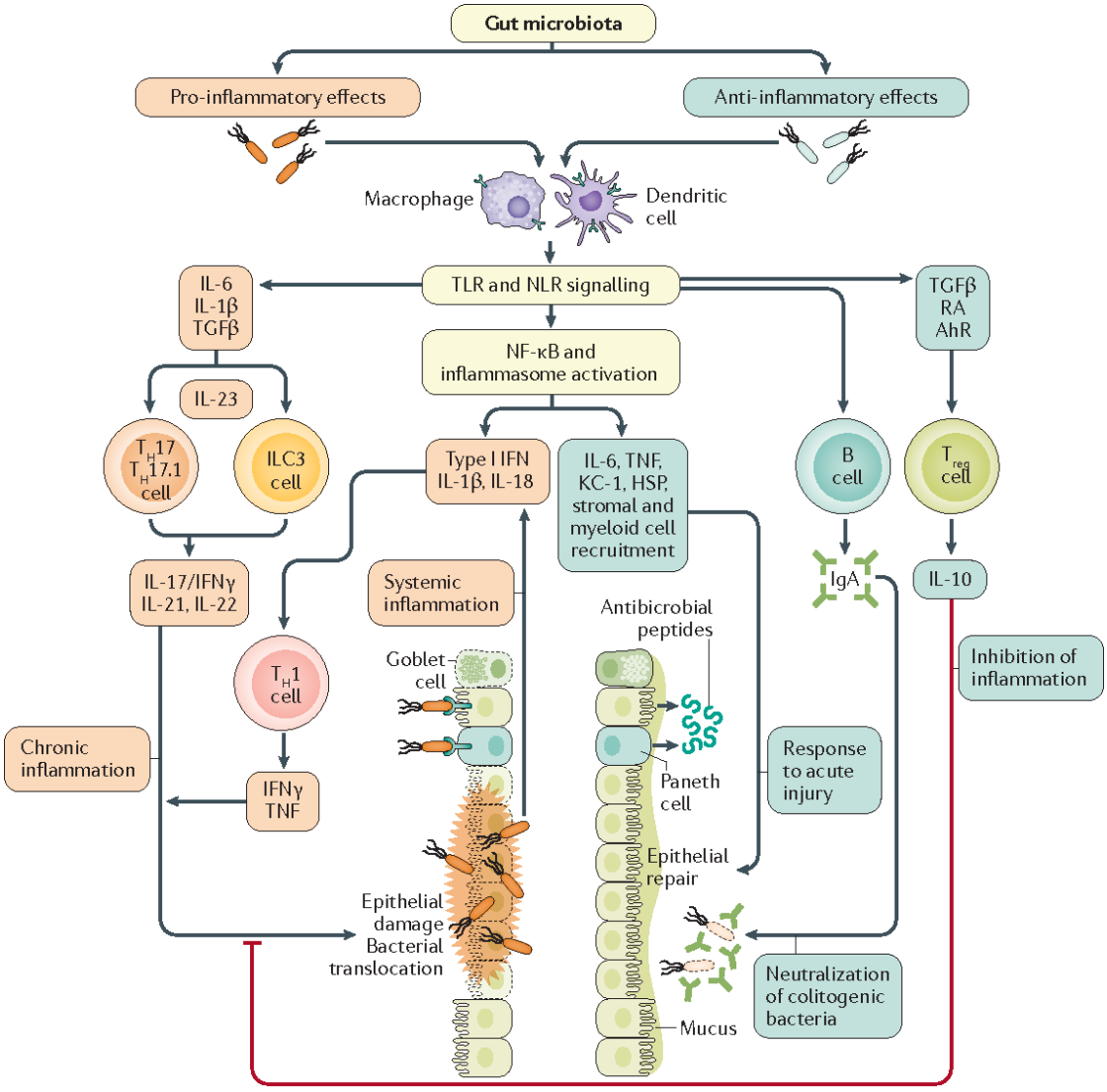

Figure 5. Mechanisms mediating the pro-inflammatory and anti-inflammatory effects of the gut microbiota. Pattern recognition receptors (PRRs) located on innate immune cells (dendritic cells and macrophages) and epithelial cells sense gut microbiota, eliciting both beneficial and harmful responses depending upon the microbial components sensed (pro- or anti-inflammatory) and the subsequent pathways activated. TLR: Toll-like receptor. NLR: NOD-like receptor. TH17: Type 17 T helper cells. ILC3s: Type 3 innate lymphoid cells. TGFβ: Transforming growth factor β. TH1: Type 1 T helper cell. Treg: Regulatory T-cell. RA: Retinoic acid. AhR: Aryl hydrocarbon receptor. From Ni *et al.*, 2017.[15]

Activation of PRRs leads to the nuclear factor κB (NF-κB) and inflammasome activation and the initiation of inflammatory pathways that contributes to epithelial damage, loss of mucus-secreting goblet cells, and bacterial translocation.[52] In turn, these inflammatory responses lead to further activation of pro-inflammatory pathways in a positive feedback loop. Non-pathogenic bacteria are also sensed via PRRs and this ultimately helps the recruitment of cells necessary for

epithelial repair and for preventing bacteria from penetrating the mucus layer. The intestinal epithelium not only tolerates MAMPs from commensal bacteria but also requires these signals for its normal functioning. In this sense, several studies have proven that the interaction of IECs with the bacterial flora shapes innate and adaptive immune responses in the lamina propria determining the correct development of the immune system (Fig. 5).[4,11,15]

PRRs include six major families of receptors: Toll like receptors (TLRs), C-type lectin receptors, nucleotide-binding oligomerization (NOD)-like receptors, RIG-I-like receptors, absent-in melanoma 2 (AIM2)-like receptors and oligoadenylate synthase (OAS)-like receptors (OLRs).[52–54]

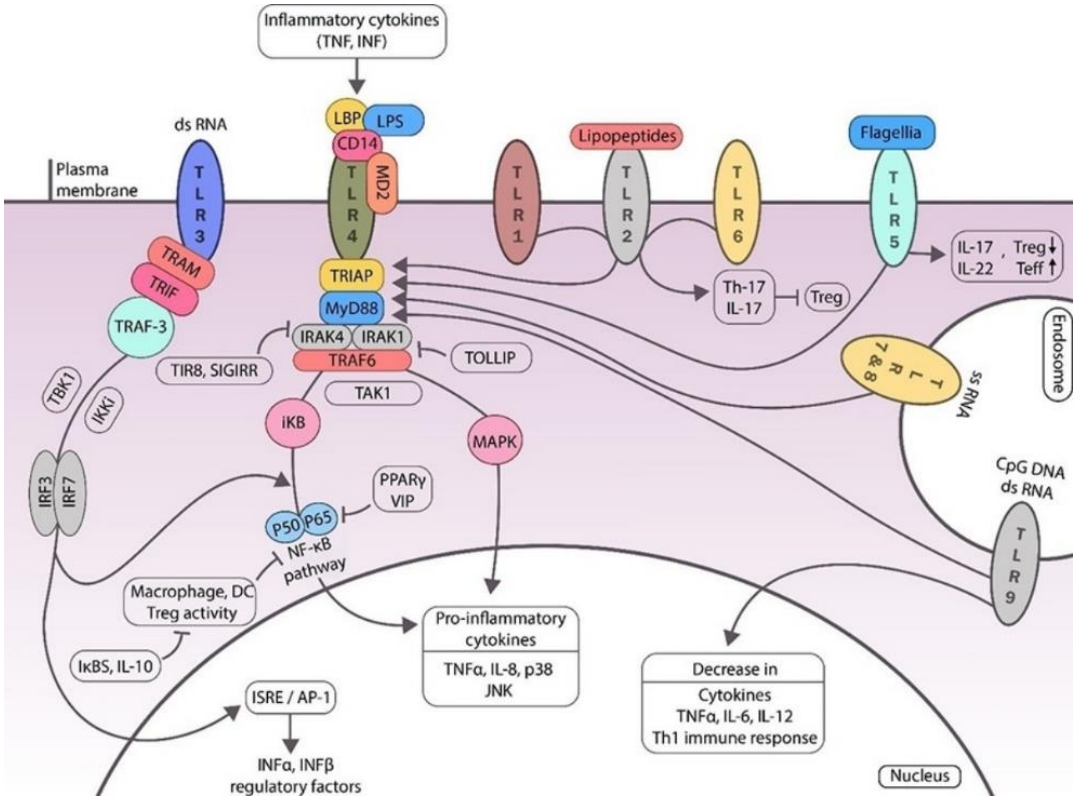

Figure 6. Schematic representation of the signaling cascades associated to the activation of TLRs by their specific ligands. TLR1 to TLR9, the more abundantly express within the GI tract, are represented. From Kordjazy *et al.*, 2018[53]. See also Table 1.

TLRs are the best characterized PRRs, and are essential in host-bacterial interactions, the maintenance of intestinal homeostasis and the development of intestinal inflammatory responses. To date, 10 human (TLR1-10) and 13 murine (TLR1-13) TLRs have been

characterized (Fig. 6; Table 1).[53] Almost all of the TLRs, at least TLR1 to TLR9, are expressed in intestinal epithelial cells, as well as in other cell types within the intestine. Given the complexity of the intestinal microbiota, careful regulation of TLR signaling must take place in order to avoid an inappropriate inflammatory response. TLRs activation induces receptor subtype-specific signaling cascades, leading to the production of cytokines and chemokines and the transcription of genes important in the control of inflammation (Figs. 5 and 6). In particular, inflammatory bowel disease (IBD) may be due to changes in TLR-dependent signaling associated to variations in receptor expression (both up-or down-regulation) and altered receptor activation due to the presence of abnormal MAMPs/PAMPs, likely due to an state of dysbiosis.[15]

Table 1. Human and murine TLRs: ligands, localization, intestinal function and expression in IBD.

	Agonist & Ligands	Localization (human and rodent)	Intestinal function	Expression in IBD
TLR1	Bacteria: Triacyl lipopeptides	Cell surface. IECs of the small intestine and colon. Dendritic cells.	Prevention of chronic inflammation and mucosal protection. Promotion of Th-17 immunity. Pro-inflammatory IL-17 T cell responses.	NC
TLR2	Bacteria: Lipoproteins/lipopeptides Peptidoglycan Lipoteichoicacid Atypical Lipopolysaccharide (LPS) Virus: Envelope protein Fungi: Zymosan Protozoa: Glycosylophosphatidylinositol	Cell surface. IECs of the small intestine and colon. Apical in villi and crypts. Low expression in adult ileum or colon, mainly in crypts. Dendritic cells.	Prevention of chronic inflammation. Promotion of colitis. Regulation of epithelial tight junctions. Regulation of chemokines and cytokines production.	NC in CD NC / ↓ in UC
TLR3	Viruses: double-stranded RNA (dsRNA)	Intracellular compartment (endosomes). IECs of the small intestine and colon.	Promotion of protective immunity under inflammatory conditions. Blockade of TLR3 reduces IL-15 production.	NC / ↑ in CD NC / ↓ in UC

	Agonist & Ligands	Localization (human and rodent)	Intestinal function	Expression in IBD
TLR4	Bacteria: LPS Virus: Envelope protein Fungi: Mannan Protozoa: Glycoinositolphospholipides Plants: Taxol	Cell surface. IECs of the small intestine and colon. Dendritic cells. Different types of immune cells.	Promotion of intestinal tissue destruction and ulceration. Protective function from invading bacteria and promotion of mucosal integrity. Regulation of cell growth, chemokine and cytokine production, phagocytosis and translocation of bacteria, and uptake of microparticles by M cells.	↑ in IBD
TLR5	Bacteria: Flagellin	Cell surface. IECs of the small intestine and colon. Dendritic cells.	Prevention of diseases associated with intestinal inflammation. Regulation of chemokines expression.	↑ in IBD
TLR6	Bacteria: D-acyl-Lipopeptides	Cell surface. Human colon and murine small intestine. Dendritic cells.	Promotion of colitis and IL-10 release.	NC
TLR7	Single-stranded RNA (ssRNA) (viral and non-viral) Bacteria: RNA	Intracellular compartment (endosomes). Human colon and murine small intestine. Dendritic cells. Lymphocytes.	Promotion of protective immunity under inflammatory conditions. Regulation of cytokine production.	↓ in IBD
TLR8	ssRNA (viral and non-viral)	Intracellular compartment (endosomes). Human colon and murine small intestine.	Induction of mucosal inflammation. Regulation of chemokines secretion.	NC in CD NC / ↑ / ↓ in UC
TLR9	Unmethylated bacterial and viral CpG DNA motifs Oligodeoxynucle-otide DNA	Intracellular compartment (endosomes). IECs of the small intestine and colon.	Enhancement of colonic barrier function. Protection against necrotizing enterocolitis. Regulation of IL-8 secretion.	NC in CD NC / ↑ in UC
TLR10	ND	ND	ND	ND
TLR11	Uropathogenic bacteria Protozoa: profilin	Cell membrane	Regulation of cytokines production	ND
TLR12	Protozoa: profilin	ND	ND	ND
TLR13	Bacteria: RNA	Intracellular compartment (probably)	Regulation of cytokine production	ND

IBD: Inflammatory bowel disease. NC: Not changed. ND: not determined. CD: Crohn's disease. UC: Ulcerative colitis.
Obtained from references 4, 53–58.

4.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are bioactive molecules synthetized and secreted locally by epithelial cells and leucocytes.[59] AMPs are one of the mechanisms by which bacterial concentrations in the small and large intestine are limited, so bacterial invasion is locally inhibited.[4,12] In addition to their antimicrobial activities, some AMPs may act as signaling molecules to host cells.[60] Gastrointestinal AMPs include defensins, C-type lectins, resistin-like molecule beta (RELM β) and cathelicidins.[59]

- Defensins:

  They are AMPs that are expressed mainly by Paneth cells or IECs, although they can be also found in some immune cells (T cells and neutrophils).[12] Defensins can be viewed as endogenous antibiotics, showing a broad microbicidal spectrum against Gram-negative and Gram-positive bacteria, fungi, viruses, and protozoa.[59] Main defensins are:

  α-defensins (or cryptidins in mice):

  The most abundant antimicrobial peptide in the human and mouse intestine, mainly found in the terminal ileum.[59–61] In CD, a reduced expression of α-defensins has been demonstrated.[12]

  β-defensins:

  Constitutively expressed at multiple sites throughout the intestine can also be induced by cytokines and bacterial products at sites of infection or inflammation.[60,61]

- C-type lectins (regenerating islet-derived protein -REG-):

  RegIIIγ is the main REG. It is produced by Paneth cells and enterocytes/colonocytes during pathogen infections or in inflammatory conditions. It is up-regulated by TLRs activation and exhibits bactericidal activity against Gram-positive bacteria.[59,61]

- Resistin-like molecule beta (RELM β):

  Bioactive molecule produced by Goblet cells that impacts immune regulation and host defense against intestinal nematode infections. It is secreted apically into the intestinal lumen and regulates susceptibility to intestinal inflammation by promoting mucosal barrier integrity (up-regulates gene expression of mucins) preventing bacteria and parasites penetration and

adhesion to the epithelium. Its secretion is known to be upregulated in several animal models of gut inflammation.[62]

- Cathelicidins:

Cathelicidins are expressed by leukocytes and IECs of the small and large intestine. They mediate the innate immune response to protect the host from infection. Cathelicidins kill bacteria by membrane insertion and disruption. They are overexpressed during inflammation and have potent anti-inflammatory effects in acute colitis.[63,64]

4.3 Cytokines

Cytokines are small, secreted glycoproteins that are produced by a wide variety of immune and non-immune cells and signal through high-affinity binding to specific cytokine receptors. Chemokines are a subfamily of cytokines that have the unique ability to direct the recruitment and migration of circulating leukocytes to specific tissues. The cytokine network is of particular relevance to gastroenterology since the gut is the main interface between the immune system and external antigens. IECs are capable of expressing a number of cytokines, which have pro- or anti-inflammatory activity, depending upon the cytokine considered. It is very clear that in IBD, there is a dramatic increase in cytokine and chemokine secretion, mainly with pro-inflammatory activity, by the epithelium, which is, largely, induced by the luminal microbiota.[65] Main pro-inflammatory immune markers involved in intestinal inflammation include the cytokines IL-6, INFɣ, TNFα, IL-18, IL-1β and IL-12p40 and the chemokine CCR5/CCL5/RANTES; on the other hand, the IL-10 is regarded as the main anti-inflammatory cytokine.

- Interleukin 6 (IL-6) is secreted by neutrophils in the early phase of inflammation and promotes monocyte–macrophage and lymphocyte recruitment. Within the intestine, IL-6 has been shown to prevent epithelial apoptosis during prolonged inflammation, having therefore a potential protective/anti-inflammatory role. Increased levels of IL-6 have been observed in IBD, both in human and in a variety of murine models of colitis.[65,66]

- Interferon-ɣ (INFɣ) is primarily secreted by Th1 T cells and NK cells. It is a potent macrophage-activating factor leading to intracellular pathogen clearance. INFɣ is increased in CD, but not UC, patients and in a number of Th1-associated mouse models of colitis.[65]

- Tumor necrosis factor α (TNFα) is produced by a variety of mucosal cells, mainly macrophages and T cells. It activates macrophages, enhancing both phagocytic activity and reactive oxygen species production. TNFα is up-regulated in patients with IBD and in several murine models of IBD.[65] TNFα and its receptors are crucially involved in the pathogenesis of IBD, as indicated by the efficacy of the anti-TNF therapy in IBD patients.[67]

- IL-1β is secreted by macrophages, monocytes and B and T cells. IL-1β transcription can be induced following stimulation of various TLR-dependent pathways and also through TNFα- or IL-1β-dependent signaling pathways. Several reports have demonstrated increased IL-1β in the inflamed gut of IBD patients, as well as in several murine models of colitis.[65]

- IL-18 is secreted mainly by macrophages, although dendritic cells (DCs) and IECs also produce it. In IBD, activated PRRs induce IL-18 secretion. In turn, IL-18 induces programmed cell death and INFɣ production which enhances local inflammation.[68]

- IL-12p40 is secreted by monocytes, macrophages and DCs. Stimulation of IL-12 receptor leads to INFɣ and TNFα production. IL-12p40 is upregulated in murine models of IBD.[65]

- CCL5/RANTES (Chemokine ligand 5/Regulated upon Activation, Normal T cell Expressed and Secreted) is a chemokine released by mast cells, activated platelets and T-lymphocytes. RANTES expression is induced by TNFα and IFNɣ. It is involved in the activation and recruitment of T cells and monocytes to the site of inflammation. Its increased expression in IBD patients and the fact that chemokine receptor 5 (CCR5)-deficient mice are less susceptible to DSS-induced colitis than their wild-type counterparts suggests the importance of CCL5/RANTES on IBD pathogenesis.[69–71]

- IL-10 is known for its broad anti-inflammatory activity on T cells, macrophages, and DCs, among other cell types.[72] Furthermore, IL-10 acts as a suppressor of Th1 responses and also can protect the epithelial monolayer from INFɣ–induced permeabilization. The production of IL-10 from IECs act as a barrier protective factor against injury resulting from the secretion of inflammatory cells.[73]

5 Functional and inflammatory gastrointestinal disorders: The microbial nexus

Functional (mainly irritable bowel syndrome; IBS) and inflammatory (inflammatory bowel disease; IBD) gastrointestinal disorders are, after cancer, the main pathophysiological alterations of the gastrointestinal tract. The gut microbiota seem to be a common pathogenic component of these alterations.[74]

5.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic immunologically mediated disease that arises as a result of a convergence of microbial factors (dysbiosis) and external environmental factors in genetically susceptible individuals (Fig. 6). IBD includes two major forms, Crohn's disease (CD) and ulcerative colitis (UC).[75] Even though they are included in the same clinical entity and share common signs, CD and UC show multiple differences in localization, symptomatology and histopathological features[76] (Table 2):

- Ulcerative colitis

  UC is typified by mucosal inflammation limited to the colon. Key features of UC include diffuse, superficial, mucosal inflammation that extends proximally from the rectum to a varying degree.[9] UC patients show a reduced number of goblet cells, a reduced thickness of the mucus layer and an altered mucus composition.[10] The clinical hallmarks of UC are diarrhea and rectal bleeding, correlating with the extent of inflammation.

- Crohn's disease

  CD is a progressive disease characterized by focal transmural inflammation affecting, potentially, any area of the intestine. The inflammation is deep and discontinuous, with distinct areas of normal tissue between affected areas, termed "skip lesions".[77] Although any site of the gastrointestinal tract may be affected, involvement of the terminal ileum is most common and the earliest mucosal lesions in Crohn's disease often appear over Peyer's patches.[9] The most common presenting symptoms of CD are diarrhea, abdominal pain and weight loss.[77]

Table 2. Clinical and epidemiological features of the two major inflammatory bowel disease subtypes.

	Crohn's disease	Ulcerative colitis
Incidence patterns		
Prevalence rates	CD is more prevalent than UC in developed countries	UC emerged before CD in developed countries, and is more prevalent in still-developing countries
Disease localization		
Affected areas	Entire gastrointestinal tract (from mouth to anus)	Colon and rectum
Inflammation pattern	Focal, patchy, discontinuous inflammation and asymmetric	Continuous inflammation in the affected area
Histopathology		
Penetrance	Transmural	Mucosal and submucosal layers
Appearance	Thickened colon wall with granulomas, deep fissures and fibrosis	Distorted crypt architecture and abscesses and shallow ulcerations
Clinical features and complications		
	Fistulas, perianal disease, abdominal mass, colonic and small-bowel obstructions, stomatitis and extraintestinal manifestations	Hematochezia, passage of mucus or pus, fulminant colitis, toxic megacolon and extraintestinal manifestations

Adapted from Lange and Barret, 2015.[76]

The exact etiology of IBD remains largely unknown. The changing epidemiology of IBD across time and geography suggests that environmental factors have a major role in inducing or modifying disease expression.[78] Environmental factors that have been consistently demonstrated in Western countries include smoking, appendectomy, diet, stress, vitamin D, and medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and antibiotics[79,80] (Fig.7).

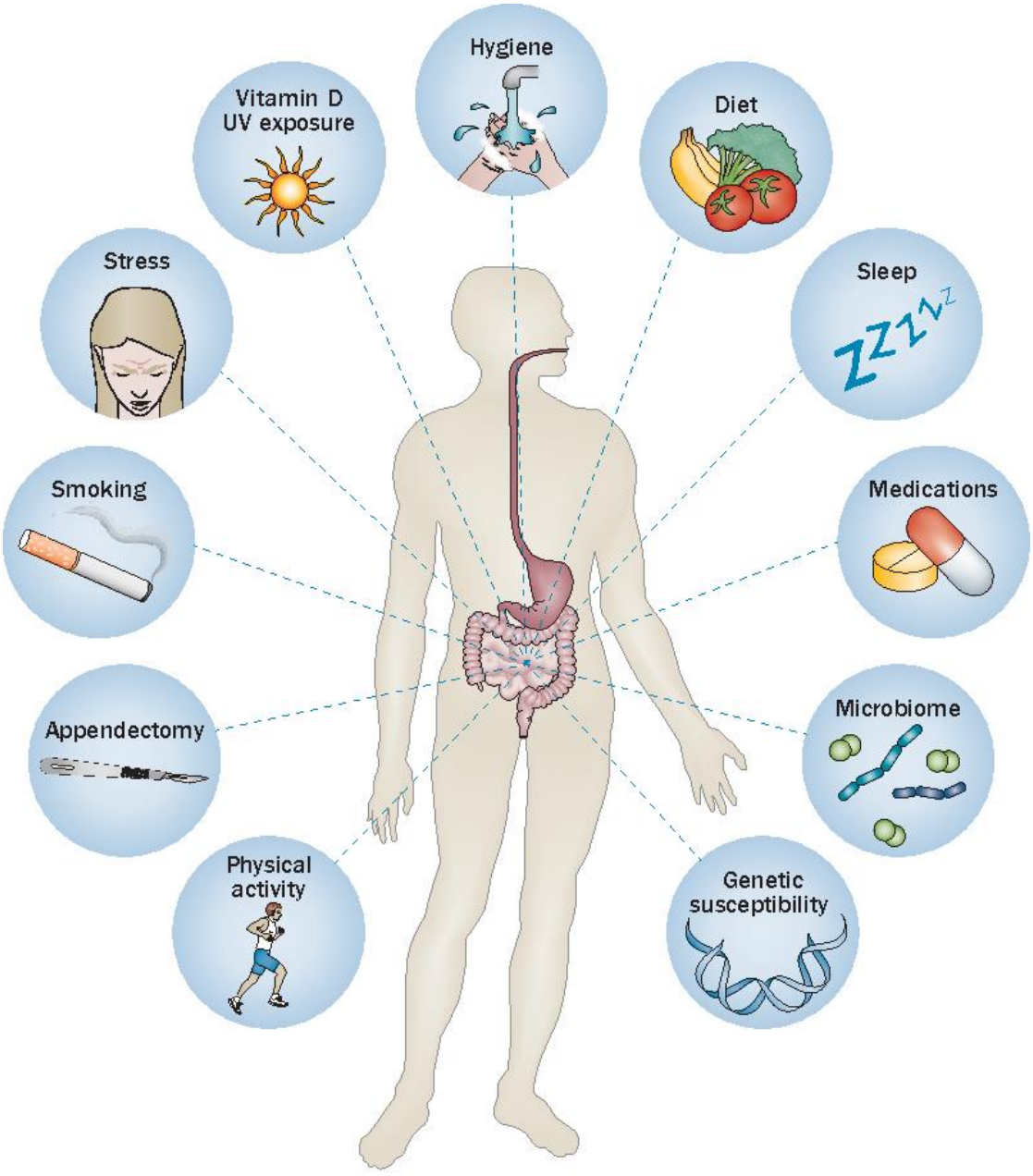

Figure 7. Important risk factors associated with IBD and disease symptoms. Despite that none of the risk factors alone is enough for developing the disease, the convergence of immunological factors, imbalanced microbiota and external environmental factors in genetically susceptible individuals leads to the development of IBD. From Ananthakrishnan *et al.*, 2018.[78]

Together with environmental factors, gut microbiota plays a central role in the development and propagation of inflammation in IBD. In IBD patients, inflammation occurs in the regions with higher bacterial density (distal ileum and colon), with a direct correlation between the severity of the colitis and the bacterial density of the intestinal mucosa.[29] It is widely documented

that IBD patients present differences in the composition of the gut microbiota (dysbiosis), particularly with respect to microbial biodiversity and the relative abundance of specific bacterial taxa, when compared to healthy individuals.[15] Although gastrointestinal dysbiosis seem to be a common finding, whether it is a cause or consequence of the disease is still debated.[7] At the same time, it is important to notice the fact that dysbiosis *per se* may not be sufficient to induce IBD;[23] although biodiversity is generally considered to be an important indicator of a healthy GCM, some species may be more important than others for maintaining immune homeostasis.[81] IBD has been associated to a decrease in biodiversity, or alpha diversity, and in species richness.[15,74,80,82–92] Descriptive analysis showed several alterations of the gut microbiota in IBD patients. In particular, changes in bacteria, including increased Veillonellaceae, Pasteurellacaea, Enterobacteriaceae, and Fusobacteriaceae, and decreased Bacteroidales, Erysipelotrichales, and Clostridiales, strongly correlated with disease status.[84,86] Particularly, in CD an increase in Bacteroidetes and Proteobacteria, concomitant with a decrease in Firmicutes abundance was described. At the species level, several butyrate-producing bacterial species (amongst them *Faecalibacterium prausnitzii*) were shown to be significantly reduced in CD patients. On the other hand, *Clostridium perfringens* was found increased in UC patients feces.[23,86] There are also specific groups of gut bacteria that may play a protective role against IBD. In this sense, a range of bacterial species, most notably the genera *Lactobacillus*, *Bifidobacterium*, and species *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, have been shown to be protective of the host from mucosal inflammation.[11,84]

There is currently no known cure for IBD, but symptoms can be managed through anti-inflammatory steroids or immunosuppressants to reduce inflammation, antibiotics to modulate intestinal microbiota, dietary changes to eliminate environmental triggers, and (in severe cases) surgery to remove damaged portions of the bowel.[76] The current gold-standard treatment is anti-TNFα antibodies, for down-regulating aberrant immune responses and inflammatory cascades. However, the adverse effects associated with these drugs and the high relapse rate limit their use and warrants investigation of novel and alternative approaches.[93]

5.2 Functional gastrointestinal disorders: Irritable bowel syndrome

IBS is a highly prevalent functional disorder characterized by abdominal pain/discomfort, bloating and altered bowel habits (with either diarrhea or constipation).[94,95]

IBS has a multifactorial etiology and pathophysiology, likely implicating alterations in gastrointestinal motility and visceral sensitivity and the functionality of the gut-brain-gut-axis.[94–96] IBS is not associated to distinct structural alterations. However, nowadays it is accepted that the disease is associated with a low-grade inflammation (without histopathological evidence) and/or an abnormal local immune response.[97]

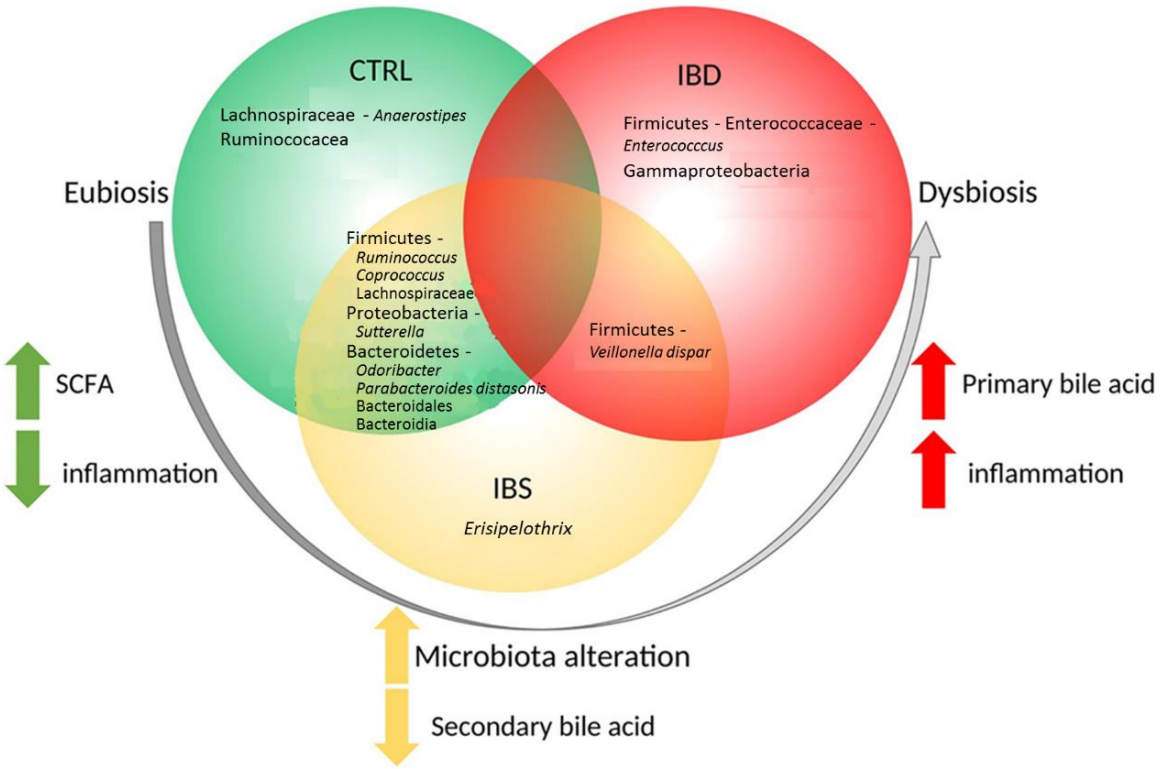

Figure 8. Model of microbiota composition and its role in the development of functional (IBS) and inflammatory (IBD) gastrointestinal disorders. The model proposed suggests that, in eubiosis conditions (CTRL, healthy subjects), specific bacterial groups compose the microbiota and maintain an equilibrium (eubiosis) characterized by production of short-chain fatty acids (SCFA) and contention on pro-inflammatory mechanisms. A microbiota alteration associated to a reduction in the production of secondary bile acids will trigger the initiation of inflammation that characterizes IBS. Then, a further increment of inflammation and the presence of primary bile acids will consolidate the dysbiotic state, which will reinforce the inflammatory reaction, leading to the development of IBD. Modified from Lo Presti *et al.*, 2019.[86]

A crucial role for the microbiota has been suggested in the pathogenesis of IBS. The most convincing evidence is the finding that IBS may develop in predisposed individuals following a bout of infectious gastroenteritis (the so-called post-infectious IBS).[97,98] Moreover, both

quantitative and qualitative changes of mucosal and fecal gut microbiota (dysbiosis) have been described in IBS patients.[86,99] IBS-associated dysbiosis implies a reduction of the diversity and stability of the microbiota, although at a lower degree to that observed in IBD.[86,100,101] In IBS patients, microbiota was characterized by an increase in the phylum Bacteroidetes and the presence of Erysipelotrichi (phylum Firmicutes, identified as a potential biomarker for IBS) and the genus *Oscillospira* (Clostridial cluster IV).[86]

Overall, and as it relates to microbiota, some authors considers that IBS represents an intermediate dysbiotic state between normal microbiota and a full-dysbiotic condition, which will correspond to IBD. The process will involve a shift in the metabolic properties of the microbiota, going from anti-inflammatory and short-chain fatty acid-producers (eubiosis) to a reduction in secondary bile acid-producers (IBS) and finally to a pro-inflammatory microbiota without secondary bile acids production (IBD with established dysbiosis)[86] (Fig. 8).

5.3 Functional and inflammatory gastrointestinal disorders and antibiotic treatments

Given the important role of the microbiota in the pathogenesis of IBD and IBS, agents that impact/modulate the gut microbiota (e.g. prebiotics, probiotics, antibiotics and fecal microbial transplant) may influence the course of these diseases. In particular, several antibiotics have been evaluated in clinical trials for the treatment of IBD and IBS, with beneficial effects.[102–104] However, to date, these approaches have not been as successful as hoped and administering systemic antibiotics remains controversial.[105] Immunomodulatory antibiotics are an interesting therapeutic strategy for intestinal inflammation, targeting both the dysbiotic microbiota and the exacerbated inflammatory response.[106] In this context, the gut-specific antibiotic rifaximin may provide an alternative to systemically delivered antibiotics and immunosuppressive agents.[104,105] In this work, we have focused on characterizing the mechanism of action of rifaximin modulating acute intestinal inflammation and inflammation-associated dysbiosis in a mouse model of colitis.

5.3.1 Rifaximin

Rifaximin (4-Deoxy-4'-methylpyrido(1',2'-1,2)imidazo(5,4-c)rifamycin SV) (Fig. 9) is a non-absorbable semisynthetic antibiotic, derived from rifamycin, with a broad-spectrum activity against gram-positive and gram-negative micro-organisms within the gastrointestinal tract.[107–109]

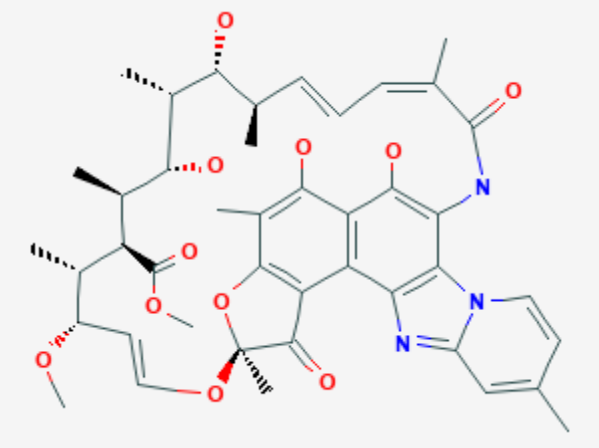

Figure 9. Chemical structure of rifaximin (CAS 80621-81-4; PubChem).

Rifaximin was initially approved by the FDA for the treatment of traveler's diarrhea caused only by noninvasive strains of *E. coli* in patients ≥12 years of age and for hepatic encephalopathy due to its inhibition of ammonia-producing enteric bacteria and consequent reduction of circulating ammonia in patients with cirrhosis.[107,110] Because of its beneficial effects in IBS patients, it was lately approved by FDA for the treatment of IBS diarrhea-predominant.[111,112] In the clinical practice, because of its theoretical ability to balance the GCM, it is often prescribed for other organic and functional GI disorders characterized by a quantitative flora alteration, such as IBD, small intestine bacterial overgrowth, and diverticular disease. Rifaximin has been tested in some clinical trials in UC and CD with beneficial effects, although these evidences remain preliminary and larger trials are needed to confirm its efficacy.[7]

The main advantage of rifaximin is that it is virtually unabsorbable (<0.4% of the dose), which minimizes systemic exposure and adverse events in all patient populations.[113] The mechanism of action of rifaximin is based on the inhibition of prokaryotic RNA-polymerase which suppresses initiation of chain formation during RNA synthesis.[114] Although its antibacterial properties, rifaximin ameliorates diarrheal disease symptoms without significant alteration of

the intestinal pathogen or the counts of intestinal flora.[107,115] Similarly, in IBD patients, despite its potent *in vitro* antibiotic activity, rifaximin administration does not appear to alter the overall composition of the gut microbiota, but seems to change the relationship between different species, modulating their abundance.[116] Due to this non-traditional property, rifaximin is often referred to as a "eubiotic".[7,117]

Current evidence shows that beneficial effects of rifaximin in IBD may not be associated to its direct bactericidal actions, but to intrinsic immunomodulatory activity. Indeed, rifaximin is able to exert an agonist activity toward the human pregnane X receptor (PXR). By activating PXR, rifaximin antagonizes TNFα on IECs, thus having an anti-inflammatory activity.[105,118]

- The pregnane X receptor

The PXR, also known as the steroid and xenobiotic sensing nuclear receptor (SXR) or nuclear receptor subfamily 1, group I, member 2 (NR1I2), is a protein that in humans is encoded by the *NR1I2* (nuclear Receptor subfamily 1, group I, member 2) gene.[119] PXR, senses and responds to a variety of chemical stimuli ranging from endogenous substances (including steroids) to drugs (such as Rifaximin) or microbial products.[119,120] PXR plays a key role in the intestinal epithelium, maintaining barrier function and enhancing wound healing through its anti-inflammatory mechanism.[121] Its primary function is to sense the presence of foreign toxic substances and to up-regulate the expression of proteins involved in the detoxification and clearance of these substances from the body. In humans, PXR is mostly expressed in the small intestine, colon and liver.[7] Gene expression profiling of inflamed colon tissues from UC and CD patients identified a significant down-regulation of PXR, suggesting a role for PXR in the pathogenesis of IBD.[122]

As mentioned, the selective activation of the PXR attenuates NFκB-dependent inflammatory signaling in the intestinal epithelium, an effect associated with protection in experimental models of colitis (Fig. 10).[121] In addition, activation of the human PXR enhanced IECs migration and accelerated intestinal epithelial wound healing[120] and preserved the barrier function of cells exposed to TNFα/INFɣ. *In vivo*, using mouse models, activation of the PXR blocked intestinal epithelial barrier disruption evoked by TLR4 activation or during dextran sulfate sodium (DSS)-induced experimental colitis.[121]

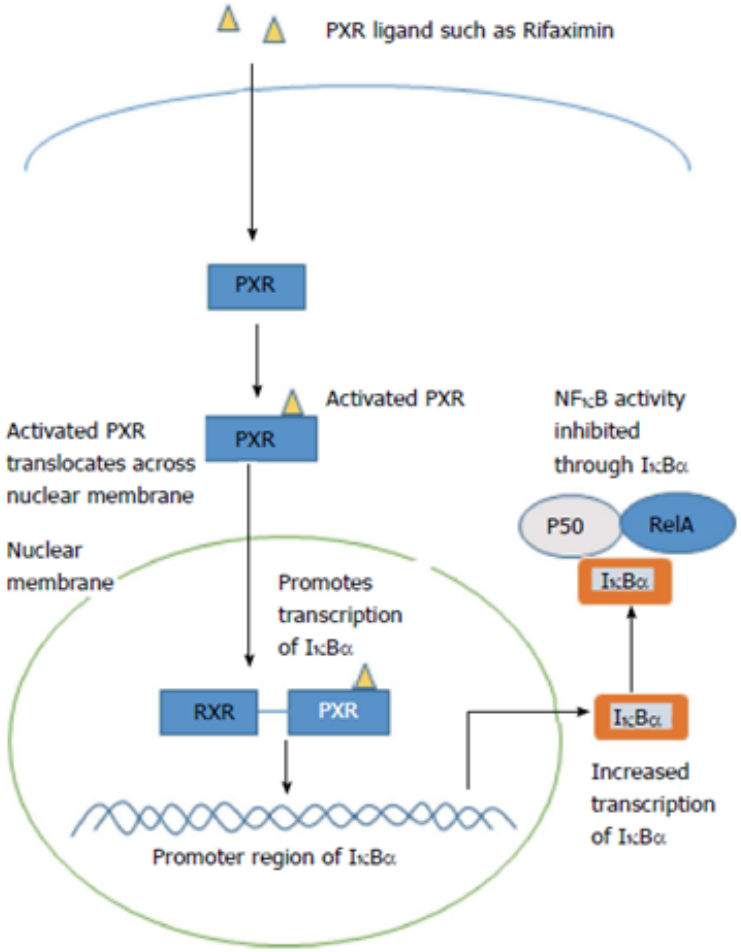

Figure 10. Anti-inflammatory effects associated to PXR activation. Upon activation by specific ligands, including rifaximin, PXRs translocate across the nuclear membrane, leading to the indirect repression of NFκB and the subsequent inhibition/attenuation of inflammatory responses. From Mohandas and Vairappan, 2017.[123]

5.3.2 Doxycycline

Doxycycline (Doxycycline hydrochloride hemiethanolate hemihydrates) is a semi-synthetic broad-spectrum second generation tetracycline (Fig. 11). Besides its antimicrobial activity, doxycycline has been reported to exert immunomodulatory activities; including modulatory effects on different immune cell populations, direct inhibition of enzymes involved in the inflammatory process (such as matrix metalloproteinases and secretory phospholipase A2), anti-apoptotic, anti-proliferative and antioxidant properties.[124,125] Several studies show that the combination of antimicrobial and immunomodulatory effects results in an effective anti-inflammatory activity in different experimental models of colitis.[106,126] Taking into consideration

these observations, in this work doxycycline was used as a positive control, having demonstrated anti-inflammatory activity during colitis.

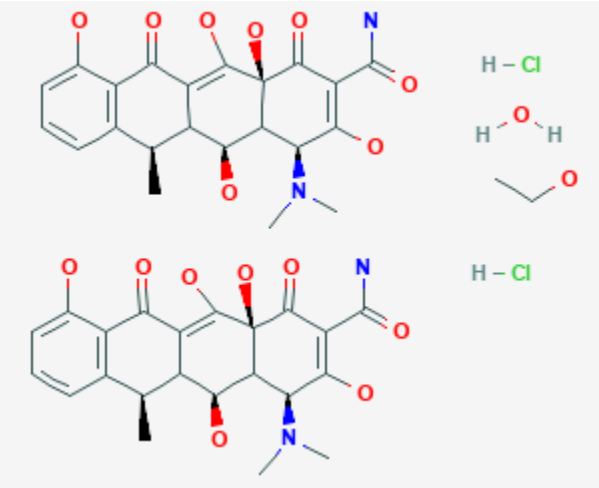

Figure 11. Chemical structure of doxycycline (CAS 24390-14-5; PubChem).

6 Animal models of intestinal inflammation

Animal models of intestinal inflammation are indispensable for our understanding of the pathogenesis of IBD and IBS in humans and the identification and validation of new therapeutic targets. The available animal models attempt to recreate the morphological, histopathological and clinical features of human IBD, and in many cases IBS, replicating different components of the disease. However, no single murine model can fully represent the set of complex features involved in human inflammatory conditions of the gut, which include host defects in epithelial barrier function, innate and adaptive immunity as well as intestinal dysbiosis. Both the pathophysiology and the location and severity of the associated lesions will vary depending on the model considered. Depending upon the model, the most relevant alterations will be found in the epithelial barrier, the innate immune response or the adaptive immune system. On the other hand, although most models are associated with colitis, inflammation of the small intestine can also be observed in some cases/models.[127–129].

Rodent (mainly in mice) models for intestinal inflammation can be classified into four major groups that are discriminated on basis of the trigger to induce inflammation: (i) spontaneous

colitis (including congenital and genetically engineered colitis); (ii) chemically-induced colitis, (iii) biological agents-induced colitis; and (iv) adoptive cell transfer colitis.[29]

- Spontaneous colitis (including congenital and genetically engineered colitis)
  Several models of spontaneous congenital intestinal inflammation have been described in mice, mainly the C3H/HeJBir mice and the SAMP1/YitFc mice. C3H/HeJBir mice develop perineal ulcers and colitis due to alterations in T lymphocyte function. SAMP1/YitFc mice are not a model of colitis, since they develop terminal ileitis due to primary epithelial barrier failure.[127,129,130]

  More than 70 different genetically engineered murine models have been described for the study of IBD/intestinal inflammation. Depending upon the genetic modification, both the etiopathogenesis and the location of the lesions differ amb both colitis and/or ileitis can be found. One of the most common genetically engineered mouse line is the IL-10-deficient mice, that develops spontaneous colitis on, at least partially, microbiota-dependent manner.[29,130]

- Chemically-induced colitis
  These models are based on the local or systemic administration of agents that, by different mechanisms, induce an inflammatory response in the intestine, often colitis. In addition to the genetic background and the housing conditions, the inflammatory response is conditioned by factors such as concentration, dose, volume, vehicle, point and route of administration of the inductor, as well as by the frequency and duration of the treatment.[127,128,130] Taking into account the route of administration of the inductor, two main groups can be distinguished: rectal and oral.

  Rectally induced colitis: acute or chronic colitis can be induced by rectally injecting a haptenating agent (usually dissolved in ethanol, to disrupt the epithelial barrier and facilitate the chemical effect). The most common agents are trinitrobenzene sulfonic acid (TNBS), dinitrobenzene sulfonic acid (DNBS) and oxazolone. These products produce an isolated point of inflammation and necrosis as well as self-antigens that provoke immune responses.[29,130]

Orally induced colitis: for oral induction, the more frequently used model is based on the administration of dextran sulfate sodium (DSS) polymers in the drinking water. Exposure to DSS results in a colitis, probably associated with the toxicity of this product at the level of the intestinal crypts, and the consequent alterations of the mucosal barrier. This allows large molecules and luminal bacteria to pass through to the lamina propria, leading to an acute inflammatory response.[4,29] This state is particularly useful to study the contribution of innate immune mechanisms, since the adaptive immune system does not play a major role in the acute inflammatory response elicited by the model.[131] Because of its consistency, this model has been widely used and it is regarded as a well-validated model of human intestinal inflammation.[132] In mice, exposure to 3-10% of DSS in the drinking water for a period between 7 and 10 days induces a colitis which is manifested by clinical signs (diarrhea, fecal blood, weight loss) and macroscopic (shortening and increased relative weight) and microscopic alterations (epithelial damage and inflammatory infiltrate) at the colon. Moreover, significant changes at the molecular level, particularly as it relates to immune-related markers can also be detected.[132–135] All these changes are reproducible and quantifiable, thus corroborating the validity of the model. In this work, and taking into account these characteristics, this has been the model selected to characterize the interplay between rifaximin and microbiota in the development of intestinal inflammation (colitis).

- Biological agents-induced colitis (transient experimental infection)

  Different biological agents (mostly pathogenic bacteria) have been used to induce a transient experimental infection as a trigger of inflammation. Bacterial pathotypes used include: enteroaggregative *E. coli*, enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, diffusely adherent *E. coli*, adherent-invasive *E. coli* (AIEC), *Clostridium difficile*, *Citrobacter rodentium*, *Salmonella enterica* serovar Typhimurium and *Campylobacter jejuni*.[29,136–139]

- Adoptive cell transfer colitis

  In these models, intestinal inflammation is induced by the transfer of a given lymphocyte subpopulation to immunodeficient receptor animals. The inflammatory response elicited (anatomical distribution and predominant immune response) will depend upon the properties of the transferred lymphocytes. This models are ideal for exploring the earliest immunological events associated with intestinal inflammation and for studying the specific

roles played by immune cell populations, including regulatory T cells, DCs and monocytes, in suppressing or limiting inflammation onset or duration.[29,127,130]

6.1 Microbiota and intestinal inflammation in rodents

Detailed knowledge on the gut microbiota composition in intestinal inflammation in rodents is still limited. Existing reports show that during DSS-induced colitis in mice, the composition of the colonic microbiota was profoundly changed and correlated with several alterations of the immune system.

As it relates to bacterial diversity, differences exists when assessing fecal samples or tissue, to assess mucosa-associated microbiota.[140] For instance, during DSS-induced colitis in mice, no changes in biodiversity were reported in colonic tissues;[140–144] however, a loss of diversity, consistent with the general believe in IBS and IBD in humans, was detected in fecal samples.[140,145]

Specific microbial changes, at al taxonomic levels, have also been described during experimental colitis in mice. However, a great variability between studies can be noticed.[141] This reveals the complexity of the gut microbiota *per se*, and their dependence on multiple modulatory factors (genetic, environmental, experimental, etc.). As a guidance, Tables 3 and 4 summarize the findings of the main studies addressing the microbiota during DSS-induced colitis in mice.

Table 3. Overview of main bacterial groups of the intestinal (ceco-colonic) microbiota enriched during DSS-induced colitis in mice.[1]

Bacterial groups affected	Reference
Enterococcus *Enterobacterium* *Staphylococcus aureus*	146
Proteobacteria (phylum) Enterobacteriales (order) *Escherichia–Shigella* *Helicobacter*	147
Enterobacteriaceae Clostridiales *Escherichia coli*	142
Proteobacteria (phylum) *Bacteroides ovatus* *Clostridium perfrigens* *Helicobacter hepaticus* *Parabacteroides distasonis* *Desulfovibrio* *Escherichia* *Helicobacter* *Clostridium* *Turicibacter* *Parabacteroides* *Bacilli* *Allobaculum* *Coprobacillus* *Bacteroides* *rc4-4* unclassified_Enterobacteriaceae unclassified_Clostridiaceae unclassified_Bacteroidaceae RF39 (order) RF32 (order) Bacteroidales (order) Erysipelotrichales (order)	140
Akkermansia *Desulfovibrio* Enterobacteriaceae	143
unclassified_Clostridiales Ruminococcaceae Bacteroidaceae Proteobacteria (phylum) Enterobacteriaceae Deferribacteraceae / *Mucispirillum* Verrucomicrobiaceae / *Akkermansia*	148
Bacillaceae Lactobacillaceae Verrucomicrobiae Enterococcales Enterobacteriaceae	149
Tenericutes Lachnospiraceae	145
Firmicutes (phylum) Bacilli (class) Lactobacillaceae	106
Bacteroidales Enterobacteriales Deferribacterales Verrucomicrobiales Erysipelotrichales	150
Bacteroidales (order) *Klebsiella* *Proteus* *Enterobacter*	151

[1:] Increased bacterial groups in animals with colitis vs. healthy animals are presented.

Table 4. Overview of main bacterial groups of the intestinal (ceco-colonic) microbiota reduced during DSS-induced colitis in mice.[1]

Bacterial groups affected	Reference
Bifidobacterium bifidus *Lactobacillus* spp.	146
Bacteroidetes (phylum) Spirochaetes (phylum) Bacteroidales (order) Clostridiales (order) *Lactobacillus*	147
Bacteroidales group *Bryantella* spp. *Tannerella* spp. Clostridia Lactobacilli	142
Bacteroidetes (phylum) *Desulfovibrio* *Oscillospira* *Odoribacter* *Coprococcus* *Dehalobacterium* *Adlercreutzia* *Lactobacillus* *Prevotella* *Bifidobacterium* unclassified_Lachnospiraceae unclassified_Lactobacillaceae unclassified_S24-7 Lactobacillales (order)	140
Lactobacillus	143
unclassified_Bacteroidales Rikenellaceae	148
Bacteroidetes *Prevotella* *Clostridium* cluster XIVa	149
Verrucomicrobia	145
Bacteroidetes (phylum) Bacteroidaceae *Bacteroides acidifaciens* Porphyromonadaceae Prevotellaceae	106
Clostridiales	150

[1:] Decreased bacterial groups in animals with colitis vs. healthy animals.

Hypothesis and Objectives

From the previous background, we can conclude that gut commensal microbiota is a key component of gastrointestinal homeostasis and has the ability to affect host immunity acting as an immune modulator. Although a definitive cause-effect relationship has not been demonstrated, compelling evidences show that microbiota plays a key role in the pathogenesis of inflammatory (IBD) and functional gastrointestinal disorders (mainly IBS). Given the well-established immunomodulatory effects of the microbiota, there is a growing interest in the manipulation of gut bacteria as a potential target for IBS and IBD therapeutics.

Thus, this work is based on the **HYPOTHESIS** that modulation of the gut commensal microbiota and, therefore, host-bacterial interactions systems, might modify intestinal inflammation.

In order to demonstrate this hypothesis, we characterized the effects of the broad-spectrum non-absorbable antibiotic, rifaximin, on gut commensal microbiota and characterized, at molecular and cellular levels, changes in immune-related markers in normal, healthy mice or during a state of colitis.

Considering this, the specific **OBJECTIVES** of this work were as follows:

- To characterize the effects of rifaximin on luminal and wall-adhered gut commensal microbiota in normal, healthy mice.
- To determine if rifaximin modulates colonic immune-related systems in normal, healthy mice.
- To evaluate the effects of rifaximin on gut commensal microbiota during intestinal (colonic) inflammation in mice.
- To assess the effects of rifaximin on the course of inflammation and colonic immune-related systems, including host-bacterial interaction systems, during intestinal inflammation (colitis) in mice.

Chapter 1

Effects of Rifaximin on Luminal and Wall-Adhered Gut Commensal Microbiota in Mice

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Abstract

Background: Rifaximin is a broad-spectrum, non-absorbable antibiotic that ameliorates symptomatology in inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) patients with both microbial dependent and independent mechanisms. **Aims**: To assess changes in gut commensal microbiota (GCM) and Toll-like receptors (TLRs) associated to rifaximin treatment in mice. **Methods**: Adult C57BL/6NCrl female mice were treated (7 or 14 days) with rifaximin (50 or 150 mg/mouse/day, PO). Luminal and wall-adhered ceco-colonic GCM were characterized by fluorescent *in situ* hybridization (FISH) and microbial profiles determined by terminal restriction fragment length polymorphism (T-RFLP). Colonic expression of TLR2, 4 and 5 and immune-related markers (IL-6, INFɣ, IL-12p40, IL-10, DEFα24, RELMβ, RegIIIɣ and IL-18) was assessed by RT-qPCR. **Results**: Regardless the period of treatment or the dose tested, rifaximin did not alter total bacterial counts (10^{10} cells/ml; vehicle: 4.3±1.1; 50 mg: 4.1±0.4; 150 mg: 4.8±1.2; $p>0.05$). Considering the GCM composition, rifaximin had a minor effect, with only a slight increase in the counts of *Bacteroides* spp. (150 mg, 1-week treatment). Bacterial diversity, as assessed by T-RFLP, was not affected. In control conditions, only *Clostridium* spp. and *Bifidobacterium* spp. were found attached to the colonic epithelium. Rifaximin showed a tendency to favour the adherence of *Bifidobacterium* spp. and *Clostridium* spp., after 1-week treatment period; no changes were observed at 2 weeks. Minor up-regulation (1- to 2-fold) in TLRs expression was observed. Only the 50 mg dose for 1 week led to a significant increase (by 3-fold) in TLR4 expression. Similarly, regardless the dose or time of treatment, no changes in the expression of other immune-related markers were observed. **Conclusions**: Results obtained show that rifaximin, even though its antibacterial properties, induces very minor changes in luminal and wall-adhered GCM in healthy mice. Moreover, no modulation of TLR-dependent host-bacterial interaction systems or local immune systems was observed. These findings, in normal conditions, do not rule out a modulatory role of rifaximin in inflammatory and or dysbiotic states of the gut.

Key words: dysbiosis, gut commensal microbiota, host-bacterial interaction systems, immune markers, rifaximin, Toll-like receptors

Introduction

Rifaximin is a semi-synthetic non-absorbable antibiotic derived from rifamycin and with a broad-spectrum activity against gram-positive and gram-negative microorganisms proposed to act on the gut microenvironment.[1,2] The main advantage of rifaximin over similar antibiotics is that it is virtually unabsorbable, which minimizes systemic exposure and adverse events in all patient populations.[3] It showed to be effective for a variety of clinical uses and was initially approved for the treatment of traveler's diarrhea caused only by noninvasive strains of *E. coli*[4] and hepatic encephalopathy (due to its inhibition of ammonia-producing enteric bacteria and consequent reduction of circulating ammonia in patients with cirrhosis).[4] Further clinical evidences led to the approval of the use of rifaximin for the treatment of diarrhea-predominant irritable bowel syndrome (IBS).[5] Moreover, in the clinical practice, rifaximin is often prescribed for other gastrointestinal disorders such as inflammatory bowel disease (IBD), small intestinal bacterial overgrowth (SIBO), and diverticular disease because of its theoretical capability to modulate the intestinal microbiota.[6]

Irritable bowel syndrome is a chronic, functional gastrointestinal disorder characterized by abdominal pain/discomfort, associated with altered bowel habits. The etiology of IBS is unknown and the pathophysiology is complex, heterogeneous, and not well understood. There is evidence for a number of underlying mechanisms, including altered intestinal barrier function, altered motility, visceral hypersensitivity and, possibly, a chronic, low-grade inflammatory or immunological response.[7–9] Moreover, interactions between environmental factors, such psychosocial stress and anxiety, led to the inclusion of brain-gut interactions in the etiology of the disease.[7,8] During the last years, gut microbiota has also been implicated in the pathogenesis of IBS. In this sense, some IBS patients have reduced gut microbial biodiversity[8], some IBS symptoms have been associated with SIBO,[7,8] and acute enteric infection have been associated to the development of IBS, the so called post-infectious IBS.[10] Overall, the presence of dysbiosis, temporal or permanent, has been seen in more than 70% of patients with IBS,[8] although great variability has been observed.[11]

The connection between dysbiosis and IBS is not completely understood, and a causal relationship has not been demonstrated.[11,12] It is known that gut commensal microbiota (GCM) contributes to the development and maintenance of gastrointestinal homeostasis.[13] Therefore, modifying the GCM is a therapeutic approach of growing interest for IBS. In this context,

rifaximin was used to treat SIBO, with the aim to act on bacteria implicated in this condition, with positive results,[14] and, as mentioned above, was approved of its use for the treatment of diarrhea-predominant IBS.[15]

Besides its antibacterial effects, pre-clinical evidences suggest that rifaximin might have anti-inflammatory activity, reducing mucosal inflammation and visceral hypersensitivity and restoring epithelial barrier function.[16–19] Whether or not these effects are secondary to its microbial actions or are direct, non-microbial-related, is still a matter of debate.

In this study, we assessed the effects of rifaximin on GCM in healthy mice. For this, we used fluorescence *in situ* hybridization (FISH) and a terminal restriction fragment length polymorphism (T-RFLP) analysis to determine rifaximin-induced changes in colonic luminal and wall adhered bacteria. To determine if changes in GCM might be associated to the local modulation of host immune-related responses, we also assessed (real-time PCR) changes in the expression of toll-like receptors (TLR)-dependent host-bacterial interaction systems and immune-related markers.

Materials and Methods

Animals

Female C57BL/6NCrl mice (n=31), 6 week-old upon arrival, were obtained from Charles River Laboratories (Lyon, France). All animals were group-housed (4-6 animals per cage) under controlled temperature (20-22ºC) and photoperiod (12:12 h light-dark cycle) and had unrestricted access to standard mouse chow and tap water. Mice were allowed to acclimatize to these conditions for a 1-week period prior to any experimentation. All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocols 1099 and 1101) and the Generalitat de Catalunya (protocols 5645 and 5646).

Antibiotic

Rifaximin (4-Deoxy-4'-methylpyrido(1',2'-1,2)imidazo(5,4-c)rifamycin SV; reference: R9904, CAS Number 80621-81-4; Sigma-Aldrich) was suspended, under sonication, in sterile PBS at a

final concentration of 50 mg/ml, then aliquoted and frozen (-20ºC) until use. Subsequent dilutions to obtain the desired concentrations were freshly made, on a daily basis, using sterile PBS. Sterile PBS was used as vehicle control.

Experimental protocols

Upon arrival, mice were randomly divided into 6 experimental groups: i) vehicle, 7-day treatment (n=4); ii) rifaximin, 7-day treatment at 50 mg/kg (n=6); and iii) rifaximin, 7-day treatment at 150 mg/kg (n=5); iv) vehicle, 14-day treatment (n=4); v) rifaximin, 14-day treatment at 50 mg/kg (n=6); and vi) rifaximin, 14 day-treatment at 150 mg/kg (n=6). Animals were dosed by oral gavage (0.2 ml/mice/day) with either sterile PBS (vehicle) or the appropriate dose of rifaximin. All treatments were performed in the morning, between 8:00 and 10:00 AM; during 7 or 14 consecutive days depending upon the experimental group considered. All animals were weighed daily at the time of dosing. Animals were euthanized 24 h after the last treatment for sample obtaining.

Samples collection

24 h after the last treatment animals were deeply anesthetized with isoflurane (Isoflo, Esteve, Barcelona, Spain) and euthanized by exsanguination through intracardiac puncture followed by cervical dislocation. A laparotomy was performed and fecal samples from the ceco-colonic region obtained and frozen immediately in liquid nitrogen. All fecal samples were stored at –80 ºC until analysis.

Thereafter, the cecum, colon, liver, spleen, thymus and adrenal glands were dissected and weighed. Tissue samples from the colon were fixed overnight in 4% paraformaldehyde or in Carnoy fixative (ethanol:chloroform:glacial acetic acid, 6:3:1, v:v:v) for histological studies. After fixing, tissues were paraffin embedded and 5 μm-tick sections obtained for either Hematoxylin-Eosin staining (4% paraformaldehyde-fixed tissues) or FISH (Carnoy-fixed tissues).

Table 1. Probes used for FISH and hybridization conditions.

Probe	Primer (5'→3')	Target	Hybridization conditions		
			Temp (ºC)	Formamide	Lysozyme
EUB338	GCTGCCTCCCGTAGGAGT	All bacteria	50		
NON338	ACATCCTACGGGAGGC	Non bacteria (negative control)	50		
BAC303	CCAATGTGGGGGACCTT	*Bacteroides* spp.	48		
EREC482	GCTTCTTAGTCAGGTACCG	*Clostridium coccoides* cluster XIVa	50		
LAB158	GGTATTAGCACCTGTTTCCA	*Lactobacillus-Enterococcus* spp	50		90 min, 37ºC
ENT-D	TGCTCTCGCGAGGTCGCTTCTCTT	Enterobacteria	50		
VER620	ATGTGCCGTCCGCGGGTT	Verrucobacteria	50	30%	
BIF164	CATCCGGCATTACCACCC	*Bifidobacterium* spp	50		

Histological evaluation

For histological examination, hematoxylin-eosin-stained sections from the colon were obtained following standard procedures. Colonic histology was assessed following procedures previously used by us.[20] A histopathological score (ranging from 0, normal, to 12, maximal alterations) was assigned to each animal. Specifically, parameters scored included: epithelial structure (0: normal; 1: mild alterations of the villi; 2: local villi destruction and/or fusion; 3: generalized villi destruction and/or fusion), structure of the crypts (0: normal; 1: mild alterations of the crypts; 2: local destruction of the crypts; 3: generalized destruction of the crypts), presence of edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria), presence of inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate). Scoring was performed on coded slides by two independent researchers.

Bacterial identification by fluorescence in situ hybridization (FISH)

For FISH, oligonucleotide probes consisted in a single strain DNA covalently linked with a Cy3 (carbocyanine) reactive fluorescent dye at the 5' end (Biomers, Ulm/Donau, Germany and Tib Molbiol, Mannheim, Germany). The bacterial groups characterized and the specific probes used are specified in Table 1.

For the assessment of luminal bacteria by FISH previously published methods were followed,[20–24] with minor modifications. Frozen fecal samples were thawed and 0.5 g of feces suspended in 4.5 ml of sterile and filtered PBS, including 2–4 glass beads (diameter 3 mm), and homogenized on a vortex mixer for 3 min. The fecal suspension was then centrifuged (1 min, 700g, 4ºC) in order to remove large particles from the suspension. From the supernatant 0.5 µl were collected and fixed in 1.5 ml freshly prepared 4% filtered paraformaldehyde solution. After overnight fixing at 4°C the fixed fecal samples were aliquoted (6 portions of 200 µl and 2 portions of 400 µl) and stored at −20°C until use. After thawing, fixed fecal samples were diluted in sterile and filtered PBS. Dilutions used were: 1600x and 800x for the EUB338-probe; 400x and 160x for the VER620-, EREC482- and BAC303-probes; 160x and 80x for the LAB158-probe; and 40x and 80x for the BIF164- and ENT-D-probes. Ten-well slides with round-shaped wells (7 mm diameter) were used. In order to enhance adhesion of fecal bacteria to the slide, slides were pre-treated by soaking them in a gelatin-suspension 2% [5ml: 0.1g gelatin, 0.01g KCr(SO4). 12H2O and miliQ water up to 100ml] for 30 min and allowed to dry at room temperature. Subsequently, 5 µl of the proper dilution was pipetted in each separate well. After drying at room temperature, the slides were fixed for 10 min using 96% ethanol (v/v). Dilutions of the probe were made in TE Buffer (10 mM Tris, 1 mM EDTA; Ambion, USA) to a concentration of 50 ng/µl and then stored at −20°C. Prior to use, the diluted probe solutions were further diluted in hybridization buffer (20 mM Tris–HCl, 0.9 M NaCl, 0.1% SDS, pH 7.2) to a concentration of 10 ng/µl and preheated at the corresponding temperature (see Table 1). Samples were hybridized in a dark moist chamber for 3 h by addition of the corresponding Cy3-labeled oligonucleotide probe. Treatments with formamide or lysozyme and hybridization temperatures were used as described, to achieve the optimal stringency (see Table 1 for details of hybridization conditions)[20–25]. Subsequently, the slides were rinsed in preheated washing buffer (20 mM Tris–HCl, 0.9 M NaCl, pH 7.2) for 30 min at the corresponding temperature (see Table 1). After briefly rinsing in milli-Q, the slides were air-dried and mounted with Vectashield-DAPI (Vector Laboratories, Peterbourgh, UK) on each well and a coverslip. The fluorescent stain 4',6-

diamidino-2-phenylindole (DAPI), which binds strongly to DNA, served as a control signal in all samples. Hybridized slides were viewed under oil immersion, using a Carl Zeiss Axioskop 40 FL epifluorescence microscope (filter for Cy3) equipped with a digital camera (Zeiss AxioCam MRm) for obtaining digital images (Zeiss AxioVision Release 4.8.1). For quantification of bacteria, 20 randomly selected fields were photographed, the number of hybridized cells counted using the CellC software,[26] and the mean value obtained. All procedures were performed on coded slides, to avoid bias.

To assess wall-adhered bacteria, hybridization of tissue samples was also performed. Sections from Carnoy-fixed paraffin-embedded tissues were deparaffinized, rehydrated, post-fixed in 4% paraformaldehyde and washed. Hybridization conditions used were essentially as described above for luminal bacteria (see Table 1 for hybridization conditions), but in this case tissue samples were incubated for 16h with the mix of hybridization buffer and the specific probe. In hybridized tissue samples, 20 randomly selected fields were photographed. Analysis of the images was performed manually by two independent researchers that observed the pictures and localized hybridized bacteria within the mucus layer or attached to the epithelial surface. A coincidence between the two observers in bacterial location in at least 3 out of the 20 pictures observed (15%) was required to decide that there was bacterial attachment to the epithelium.[20,23,24] All procedures were performed on coded slides, to avoid bias.

Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial communities was performed following methods published elsewhere.[21,27] Briefly, a 1497-pb fragment of the 16S rDNA gene was amplified using a 6-carboxy-fluorescein-labeled forward and reverse primers (S-D-Bact-0008-a-S-20: 5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3'; and PH1552: 5'AAGGAGGTGATCCAGCCGCA-3', respectively) against the first 20 bases of the 16S RNA sequence. Duplicate PCR were performed for each sample. Fluorescent-labeled PCR products were purified on QIAquick PCR purification kit columns (Qiagen, West Sussex, UK) and eluted in a final volume of 30 µl of Milli-Q water. Then, the resultant PCR product was subjected to a restriction with HhaI (20,000 U/µl) (Biolabs Inc., New England, USA). Fluorescent-labeled terminal restriction fragments (TRF) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan

mode with a 25-U detection threshold. Determination of the TRF sizes in the range 50–700 bp were performed with the size standard GS-1000-ROX (PE Biosystems).

Data obtained consisted of size (base pairs) and peak area for each TRF. To standardize the DNA loaded on the capillary, the sum of all TRF peak areas in the pattern was used to normalize the peak detection threshold in each sample. Following the method described by Kitts,[28] a new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total area in the sample with the smallest total peak area) by 323 area units (the area of the smallest peak at the 25 detection threshold in the sample with the smallest total peak area). For each sample, peaks with a lower area were deleted from the data set. Thereafter, a new total area was obtained by the sum of all the remaining peak areas in each pattern.

Biodiversity (also known as richness) was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated, and dendrograms were constructed using the Fingerprinting II software (Informatix, Bio-Rad, CA, USA) and an unweighted pair-group method with averaging algorithm. To deduce the potential bacterial composition of the samples, in silico restrictions for the major rat gut bacteria with the primers and the enzyme used were obtained by using the analysis function TAP–T-RFLP from the Ribosomal Database Project II software. Results are presented as potential compatible bacterial species. Note also that direct attribution of species to individual peaks is not unequivocally possible unless fingerprinting is complemented with sequence analysis of clone libraries. Analysis of electropherograms was used for the visual comparison of compatible TRF with different bacteria for the different experimental groups.

Colonic expression of TLRs and immune-related markers using quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from colonic tissue samples using Tri reagent with Ribopure Kit (Ambion/Applied biosystems, Foster City, CA, USA). RNA samples were converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA concentration was measured using NanoDrop (ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) and all the samples were diluted at 100 ng/μl with DEPC-Treated water (Ambion/ Applied biosystems, Foster City, CA, USA). TaqMan gene expression assays for interleukin 6 (IL-6) (Mm00446190_m1), interferon gamma (IFNγ)

Mm01168134_m1, interleukin-12 p40 (IL-12p40) (Mm00434174_m1), interleukin-10 (IL-10) (Mm00439614_m1), interleukin 18 (IL-18) (Mm00434225_m1), defensin alpha 24 (Defα24) (Mm04205950_gH), resistin-like molecule-β (RELMβ) (Mm00445845_m1), regenerating islet-derived protein 3 gamma (RegIIIγ) (Mm00441127_m1) and TLR2 (Mm00442346_m), TLR3 (Mm01207404_m1), TLR4 (Mm00445273_m1), TLR5 (Mm00546288_s1) and TLR7 (Mm00446590_m1) were used (Applied Biosystems). All samples, as well as the negative controls, were assayed in triplicates. β-2-microglobulin (β2m) (Mm00437762_m1) was used as endogenous control.

The PCR reaction mixture was incubated on the Bio-Rad CFX384 (Bio-Rad Laboratories). Bio-Rad CFX Manager 3.1 software was used to obtain the cycle threshold for each sample. All data was analyzed with the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the vehicle groups serving as calibrator.[29]

Statistical analysis

Data are expressed as mean ± SEM or media (interquartile range) ± SD, as indicated. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Data were analyzed by one-way analysis of variance (ANOVA), followed, when necessary, by a Student-Newman-Keuls multiple comparisons test. Data were considered statistically significant when $p<0.05$. All statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA).

Results

Effects of rifaximin on body weight, weight of body organs and colonic histology

Body weigh was stable over the treatment period, without treatment-related significant changes (Table 2). At necropsy, the relative weight of the liver was slightly, but significantly reduced in rifaximin-treated animals, in similar proportion regardless the dose and the duration of treatment ($p<0.05$ vs. respective vehicle-treated group; Table 2). Relative weight of the spleen, thymus, and adrenal glands were similar across groups.

At necropsy, no macroscopic alterations were observed in the colon or cecum, irrespective of the experimental group considered. Similarly, cecal and colonic content had a normal consistency in all experimental groups. Colonic and cecal relative weight was similar across groups (Table 2). Consistent with these observations, no histological alterations were observed in the colon in rifaximin-treated animals, regardless the treatment time or dose. In all cases, histological scores ranged between 0 and 2 (data not shown).

Table 2. Body weight and relative weight of body organs in the different experimental groups.[1]

Treatment duration	Treatment	n	Body weight at necropsy (g)	Colon (mg/cm)	Cecum (mg/g)	Spleen (mg/g)	Thymus (mg/g)	Adrenal glands (mg/g)	Liver (mg/g)
7-day	Vehicle	4	17.6±0.6	23.2±1.7	21.5±0.9	3,5±0.2	3,1±0.2	0,2±0.0	50,1±1.3
	Rifaximin (50 mg/kg)	6	17.8±0.22	21.8±0.8	25.1±2.3	2,8±0.1	3,2±0.2	0,2±0.0	44,1±0.7 *
	Rifaximin (150 mg/kg)	5	18.3±0.2	21.3±1.6	25.9±0.9	3,0±0.1	3,0±0.1	0,2±0.0	44,0±0.5 *
14-day	Vehicle	4	17.4±0.7	21.7±0.2	22.2±0.8	3,4±0.2	3,3±0.1	0,2±0.0	50,1±0.4
	Rifaximin (50 mg/kg)	6	18.3±0.3	22.9±1.3	23.7±1.9	3,1±0.0	2,8±0.2	0,2±0.0	44,2±1.1 *
	Rifaximin (150 mg/kg)	6	18.2±0.2	24.5±0.7	21.2±0.9	3,2±0.1	3,1±0.1	0,2±0.0	45,3±0.8 *

[1]: Data represent mean ± SEM of the number of animals indicated (n). *: $p<0.05$ vs. corresponding vehicle.

Effects of rifaximin on luminal GCM

In vehicle-treated animals, regardless the duration of treatment, total bacteria counts within the luminal content (EUB338-probe) oscillated between $2x10^{10}$ cells/ml and $1.5x10^{11}$ cells/ml (Figs. 1 and 2); consistent with previous observations.[20,23,30] There was a good coincidence between total bacterial counts assessed by FISH (EUB338-probe) and DAPI staining (Figs. 1 and 2). The most abundant bacterial group was *Clostridium* spp. (EREC482-probe), being within the order of 10^{10} cells/ml; followed by *Bacteroides* spp. (BAC303-probe) at 10^9 cells/ml and Verrucobacteria (VER620-probe) at 10^8 cells/ml. On the other hand, *Lactobacillus/Enterococcus* spp. and *Bifidobacterium* spp. were ranging between detection levels (10^6 cells ml) and 10^8-10^9 cells/ml. Enterobacteria appeared below or at the threshold of detection levels (Figs. 1, 2 and 3).

Total bacterial counts remained stable after the treatment with rifaximin, regardless the dose (50 or 150 mg/kg/day) or the duration of treatment considered (7-day or 14-day). Total counts oscillated between $2x10^{10}$ cells/ml and $7x10^{10}$ cells/ml and $1.5x10^{10}$ cells/ml and $1.5x10^{11}$ cells/ml for the 7-day and 14-day treatment period, respectively (Figs. 1 and 2). Assessment of specific bacterial groups showed an increase in *Bacteroides* spp. (BAC303-probe) counts after a 7-day treatment period, but not after a 14-day period (Figs. 1, 2 and 3). Although not significant, during the 14-day treatment, a dose-dependent reduction in the proportion of *Clostridium* spp. (EREC482-probe) was observed, at the expense of an increase in the relative abundance the other bacterial groups assessed, particularly *Bacteroides* spp. (BAC303-probe) (Fig. 3).

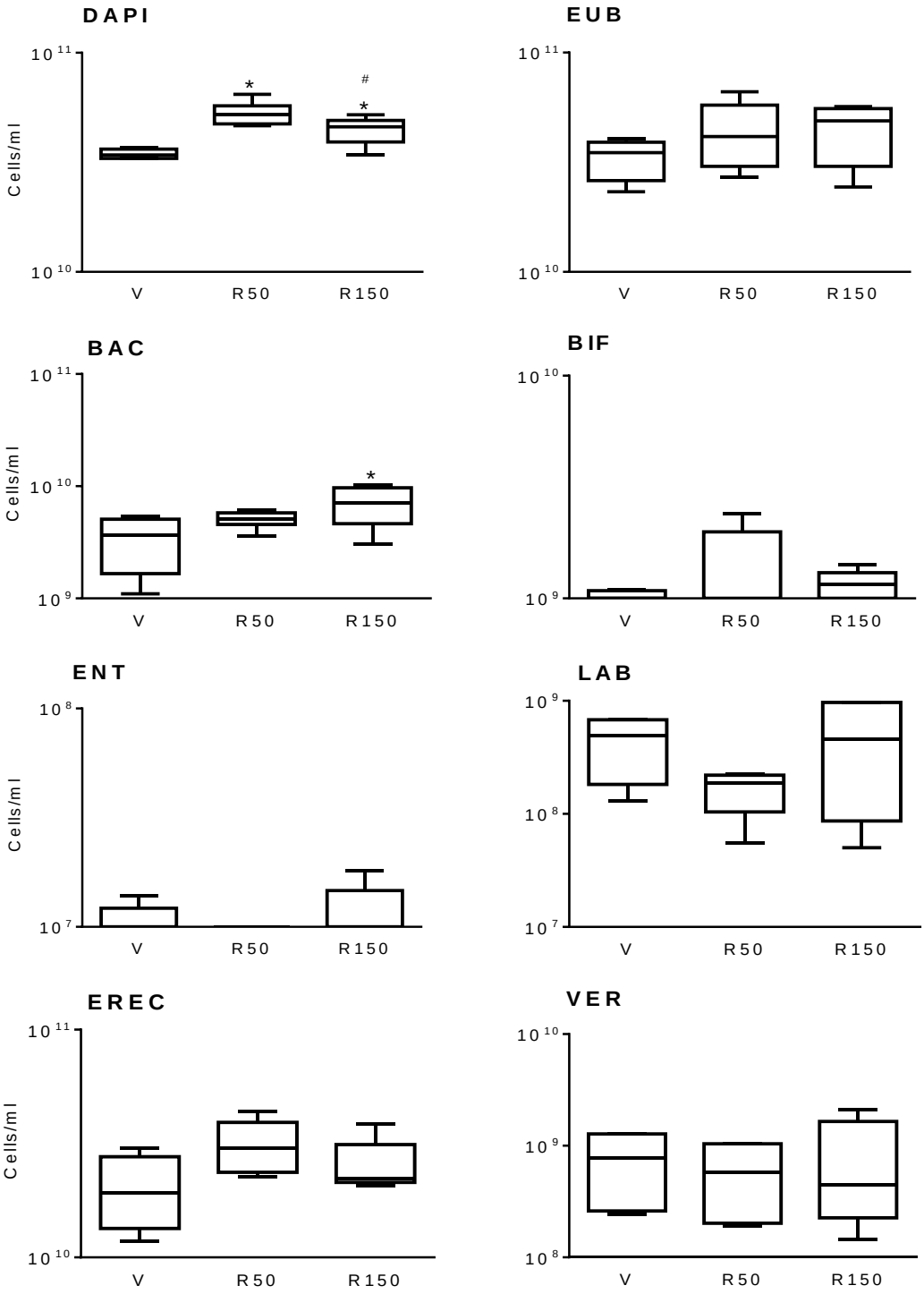

Figure 1. Colonic microbiota, as quantified by FISH after a 7-day treatment period with rifaximin. EUB: Total bacteria; BAC: *Bacteroides* spp.; ENT-D: Enterobacteria group; VER: Verrucobacteria group; BIF: *Bifidobacterium* spp.; LAB: *Lactobacillus/Enterococcus* spp.; EREC: *Clostridium* spp. cluster XIVa group. Data are media (interquartile range) ± SD, n=4-6 per group. *: $p<0.05$ vs. corresponding vehicle. #: $p<0.05$ vs. rifaximin at 50 mg/kg. V: Vehicle; R50: rifaximin at 50 mg/kg; R150: rifaximin at 150 mg/kg.

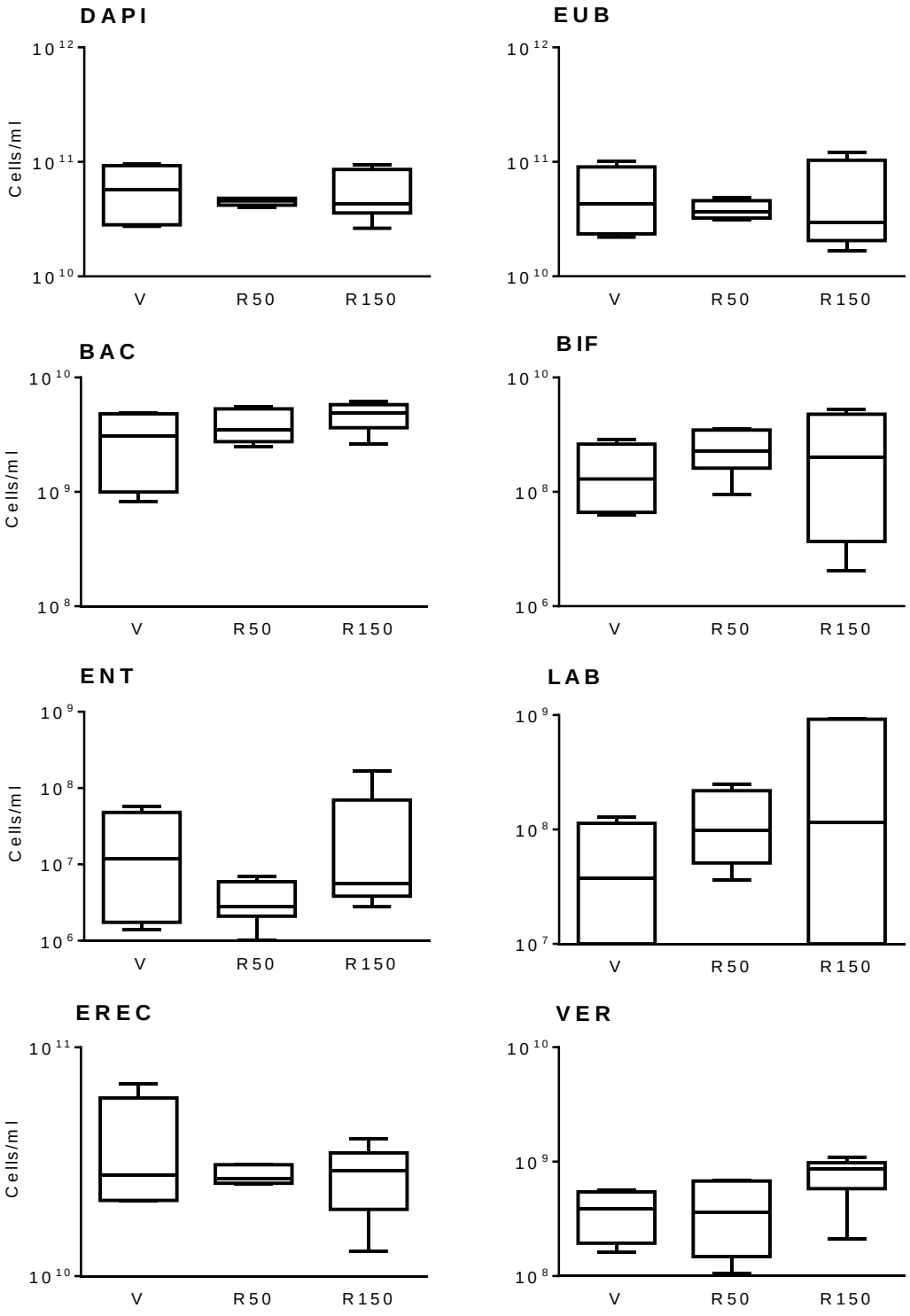

Figure 2. Colonic microbiota, as quantified by FISH after a 14-day treatment period with rifaximin. EUB: Total bacteria; BAC: *Bacteroides* spp.; ENT-D: Enterobacteria group; VER: Verrucobacteria group; BIF: *Bifidobacterium* spp.; LAB: *Lactobacillus/Enterococcus* spp.; EREC: *Clostridium* spp. cluster XIVa group. Data are media (interquartile range) ± SD, n=4-6 per group. V: Vehicle; R50: rifaximin at 50 mg/kg; R150: rifaximin at 150 mg/kg.

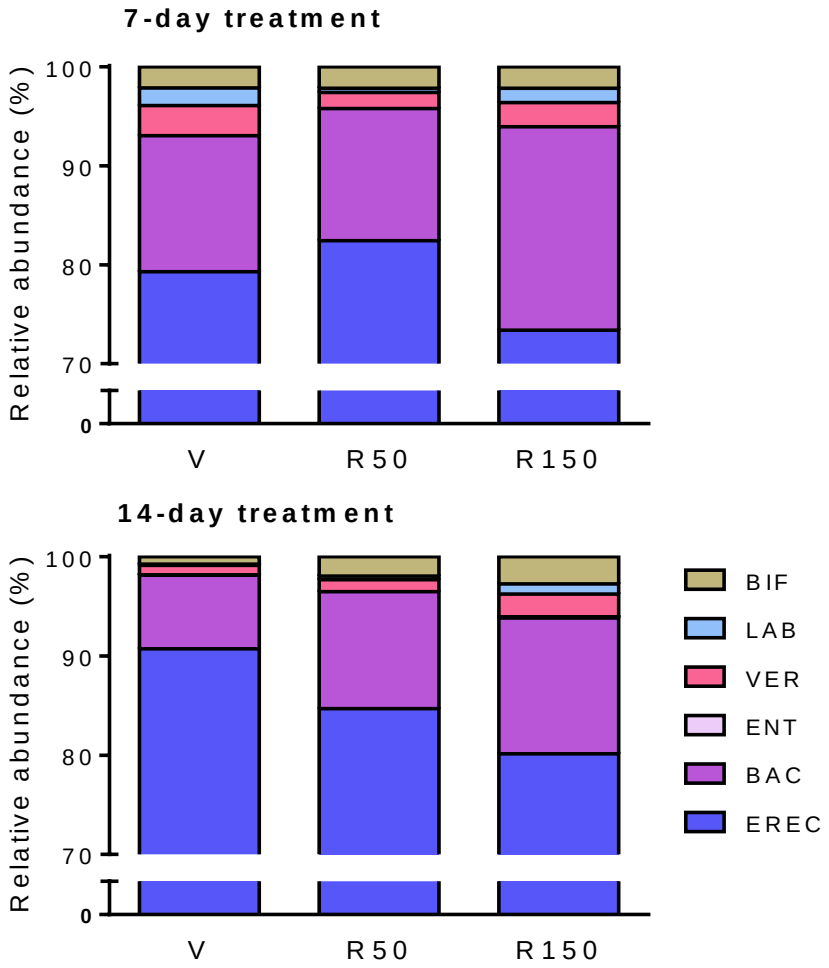

Figure 3. Relative distribution of the colonic microbiota, as quantified by FISH, in the different experimental groups. Data represent the relative abundance (percent) of the bacterial groups characterized [*Bacteroides* spp. (BAC), *Bifidobacterium* spp. (BIF), *Clostridium* spp. (EREC), Enterobacteria (ENT), *Lactobacillus* spp. (LAB), and Verrucobacteria (VER)]. Relative percent composition of the microbiota was calculated taking as 100% the total counts of the bacterial groups assessed. n=4-6 per group. V: Vehicle; R50: rifaximin at 50 mg/kg; R150: rifaximin at 150 mg/kg.

The ecological characterization of the luminal microbiota was performed with a T-RFLP analysis. The dendrogram representation of the similarity indexes of the T-RFLP profiles of the ceco-colonic microbiota did not show a clustering of the different experimental groups (Fig. 4A). The number of t-RFs and their size distribution, taken as a measure of biodiversity, was similar across experimental groups, regardless the treatment applied (P=0.1663; Figs. 4B and 5).

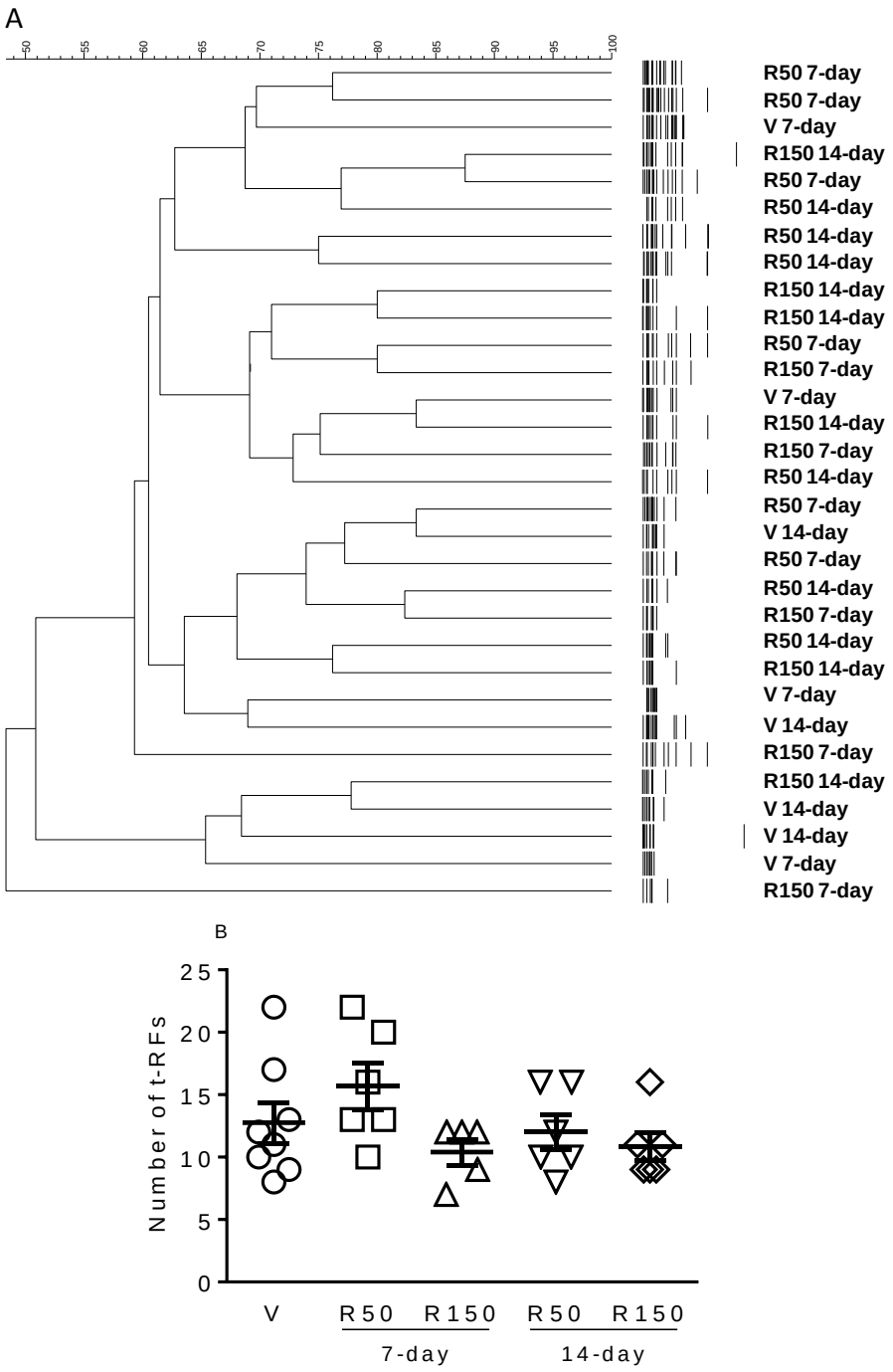

Figure 4. Ecological characterization of the luminal microbiota by T-RFLP analysis. A: Dendrogram showing the distribution of the different experimental groups according to the T-RFLP banding patterns obtained from the analysis of the ceco-colonic samples. Each line represents an animal identified by either R50 (rifaximin at 50 mg/kg), R150 (rifaximin at 150 mg/kg) or V (vehicle), followed by the experimental period (7-day or 14-day). The dendrogram distances represent percentage of similarity. The different experimental groups did not cluster together indicating that the antibiotic treatment did not modify the microbiota composition. **B:** Effects of rifaximin on biodiversity (number of tRFs detected) of the ceco-colonic microbiota. For the sake of clarity, and since no differences were observed among them, vehicle-treated animals have been merged in a single group. V: Vehicle; R50: rifaximin at 50 mg/kg; R150: rifaximin at 150 mg/kg. Data are mean ± SEM, n=6-8.

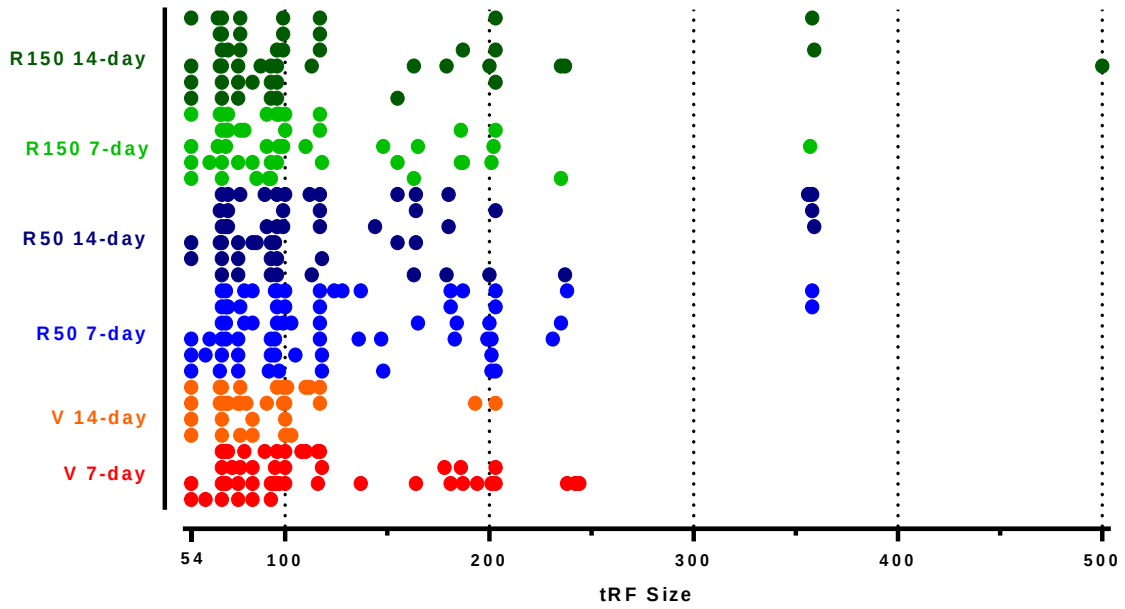

Figure 5. Ecological characterization of the luminal microbiota by t-RFLP analysis. The figure shows the distribution of the tRFs detected according to their size. Each line represents an individual animal and each column a tRF size. tRF distribution indicates a similar microbial biodiversity in all experimental groups, regardless the treatment applied. See also Table 3 for details regarding taxonomical classification of the different tRFs.

Table 3 summarizes the main bacterial groups, as detected by the t-RFLP analysis, with differential presence in the six experimental groups (see also Fig. 5 for distribution of the different tRF detected in function of their size). Overall, the t-RFLP analysis reveals high similarities in bacterial composition among the different experimental groups, without evident treatment-related changes in the diversity of the microbiota. Although similar bacterial groups were detected (according to the theoretical restriction 5'-fragment size), in many cases these groups could not be identified phylogenetically and were classified as "unidentified" or "uncultured bacterium". According to the t-RFLP, tRFs with a size between 356 and 359 appeared in some rifaximin-treated animals, regardless the dose or the duration of treatment (Fig. 5). Although this might indicate some treatment-related effect, the low incidence observed (17-33%; Table 3) complicates its interpretation.

Table 3: Theoretical restriction 5'-fragment (tRF) size predicted for the major mouse gut bacteria and prevalence in the different experimental groups.

Compatible bacterial group	tRF size	Frequency[a]					
		V 7-day (n=4)	V 14-day (n=4)	R50 7-day (n=6)	R50 14-day (n=6)	R150 7-day (n=5)	R150 14-day (n=6)
Unidentified	54–55	3 (75)	4 (100)	4 (67)	3 (50)	3 (60)	5 (83)
Bacillus spp. / *Lactococcus lactis* spp.	61–62	1 (25)	2 (50)	1 (17)	0 (0)	1 (20)	1 (17)
Salinicoccus roseus	63	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Burkholderia spp. / *Bordetella* spp. / *Thiomonas* spp. / uncultured bacterium	67	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	1 (17)
Mycobacterium spp. / uncultured rumen bacterium	68	0 (0)	2 (50)	1 (17)	2 (33)	1 (20)	2 (33)
Uncultured rumen bacterium	69	4 (100)	4 (100)	5 (83)	5 (83)	4 (80)	6 (100)
Uncultured rumen bacterium / *Leptotrichia* spp.	71	2 (50)	1 (25)	4 (67)	1 (17)	2 (40)	0 (0)
Uncultured rumen bacterium	72	1 (25)	1 (25)	1 (17)	3 (50)	2 (40)	1 (17)
Photorhabdus sp.	74–75	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Uncultured bacterium	77	2 (50)	1 (25)	3 (50)	3 (50)	1 (20)	5 (83)
Uncultured rumen bacterium / naphthalene-utilizing bacterium	78	1 (25)	3 (75)	1 (17)	1 (17)	1 (20)	3 (50)
Uncultured bacterium	80	1 (25)	0 (0)	2 (33)	0 (0)	1 (20)	0 (0)
Sphingomonas spp. / uncultured bacterium	81	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)
Uncultured bacterium	84	3 (75)	2 (50)	2 (33)	1 (17)	0 (0)	1 (17)
Desulfovibrio defluvii / *Roseiflexus* spp.	86–87	2 (50)	1 (25)	1 (17)	2 (33)	1 (20)	5 (83)
Flavobacterium psychrophilum	88–89	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)
Flavobacterium johnsoniae	90	1 (25)	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)
Anaeromyxobacter dehalogenans / uncultured bacterium	91	0 (0)	1 (25)	0 (0)	1 (17)	2 (40)	0 (0)
Geobacter spp. / uncultured Bacteroidetes / *Cytophaga* spp. / *Algoriphagus* spp.	92	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Flavobacteriaceae bacterium / uncultured rumen bacterium/ *Desulfovibrio* spp.	93–94	2 (50)	0 (0)	3 (50)	4 (67)	2 (40)	5 (83)
Desulfovibrio profundus / uncultured bacterium	95	2 (50)	0 (0)	3 (50)	1 (17)	1 (20)	0 (0)
Uncultured bacterium	96	1 (25)	1 (25)	3 (50)	4 (67)	2 (40)	4 (67)
Desulfococcus oleovorans / *Desulfomonile limimaris* / *Helicobacter pylori*	97–98	1 (25)	0 (0)	4 (67)	4 (67)	2 (40)	4 (67)

Compatible bacterial group	tRF size	Frequency[a]					
		V 7-day (n=4)	V 14-day (n=4)	R50 7-day (n=6)	R50 14-day (n=6)	R150 7-day (n=5)	R150 14-day (n=6)
Helicobacter pylori / uncultured rumen bacterium	99		2 (50)	1 (17)	2 (33)	1 (20)	5 (83)
Bacteroides spp. / uncultured rumen bacterium	100	3 (75)	3 (75)	2 (33)	1 (17)	2 (40)	0 (0)
Bacteroides fragilis / uncultured rumen bacterium / *Prevotella ruminicola*	101–102	1 (25)	2 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Uncultured rumen bacterium	103–104	1 (25)	1 (25)	1 (17)	0 (0)	0 (0)	0 (0)
Uncultured bacterium	105	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Desulfitobacterium hafniense	107–108	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Thiobacillus spp.	110–111	1 (25)	2 (50)	0 (0)	0 (0)	1 (20)	0 (0)
Uncultured bacterium	112	0 (0)	1 (25)	0 (0)	1 (17)	0 (0)	0 (0)
Unidentified	113	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (17)
Uncultured bacterium	116	2 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Unidentified	117	1 (25)	2 (50)	4 (67)	3 (50)	2 (40)	5 (83)
Desulfitobacterium hafniense	118	1 (25)	0 (0)	2 (33)	1 (17)	1 (20)	0 (0)
Unidentified	123–124	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Unidentified	127–129	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Unidentified	136	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Uncultured rumen bacterium	137	1 (25)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Microbacterium spp.	144–145	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)
Leucobacter spp. / *Janibacter* spp.	147	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Unidentified	148–149	0 (0)	0 (0)	2 (33)	0 (0)	1 (20)	0 (0)
Pseudomonas aeruginosa	155	0 (0)	0 (0)	0 (0)	1 (17)	1 (20)	1 (17)
Unidentified	156	1 (25)	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)
Unidentified	163	0 (0)	0 (0)	0 (0)	1 (17)	1 (20)	1 (17)
Synechococcus spp.	164	1 (25)	0 (0)	0 (0)	3 (50)	0 (0)	0 (0)
Unidentified	165–167	0 (0)	0 (0)	3 (50)	0 (0)	1 (20)	0 (0)
Uncultured bacterium	178	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Uncultured rumen bacterium	179	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (17)
Uncultured rumen bacterium	180	0 (0)	0 (0)	0 (0)	2 (33)	0 (0)	0 (0)
Uncultured rumen bacterium	181–182	1 (25)	0 (0)	2 (33)	2 (33)	0 (0)	0 (0)
Uncultured rumen bacterium	183	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Uncultured rumen bacterium	184–185	1 (25)	0 (0)	2 (33)	0 (0)	1 (20)	0 (0)
Listeria monocytogenes	186	1 (25)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)
Uncultured rumen bacterium	187	1 (25)	0 (0)	1 (17)	0 (0)	1 (20)	1 (17)
Uncultured rumen bacterium	193	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)

Compatible bacterial group	tRF size	Frequency[a]					
		V 7-day (n=4)	V 14-day (n=4)	R50 7-day (n=6)	R50 14-day (n=6)	R150 7-day (n=5)	R150 14-day (n=6)
Psychrobacter spp. / uncultured bacterium / *Francisella* spp.	194–195	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Unidentified	199	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Bacillus spp.	200	0 (0)	0 (0)	1 (17)	1 (17)	0 (0)	0 (0)
Clostridium rectum / uncultured bacterium / *Mycobacterium* spp.	201	1 (25)	0 (0)	3 (50)	0 (0)	1 (20)	1 (17)
Fervidobacterium spp. / *Dehalococcoides* spp. / uncultured bacterium	202	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)
Uncultured rumen bacterium	203–204	2 (50)	1 (25)	3 (50)	1 (17)	1 (20)	5 (83)
Clostridium spp.	231–232	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Clostridium perfringens	234–235	0 (0)	0 (0)	1 (17)	0 (0)	1 (20)	1 (17)
Clostridium botulinum	237	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (17)
Bacillus subtilis subsp. *Subtilis*	238	1 (25)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Bacillus subtilis subsp. *subtilis* / *Bacillus licheniformis* / *Bacillus* spp.	241–242	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Geobacillus stearothermophilus / *Paenibacillus* spp.	244	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Microbispora spp. / *Pseudonocardia compacta* / *Nonomuraea bangladeshensis* / *Kineosporia aurantiaca*	356	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)
Microbispora spp. / *Herbidospora* spp.	357	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)
Kribbella spp. / *Actinomadura* spp. / *Pseudonocardia* spp. / *Anaplasma marginale*	358	0 (0)	0 (0)	2 (33)	2 (33)	0 (0)	1 (17)
Arthrobacter spp.	359	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (17)
Uncultured bacterium	500	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)

[a]Data represent the number of animals within each group presenting the bacterial group predicted by the corresponding tRF size and the incidence, in percentage (between brackets).

Effects of rifaximin on bacterial adherence to the colonic wall

In vehicle-treated mice, *Bifidobacterium* spp. and *Clostridium* spp. were the only bacterial group attached to the colonic wall (epithelium). The overall incidence of attachment ranged from 25% to 50% and was similar after a 7-day or a 14-day treatment period (Figs. 6 and 7).

During antibiotic treatment for a 7-day period, there was a tendency to increase the incidence of bacterial wall adherence for *Clostridium* spp. (from an incidence of 25% in control conditions to 67% and 75% at 50 mg/kg and 150 mg/kg, respectively) and *Bifidobacterium* spp. (from an incidence of 25% in control conditions to 50% for both antibiotic-treated groups). However, this tendency disappeared in animals treated for a 14-day period (Figs. 6 and 7).

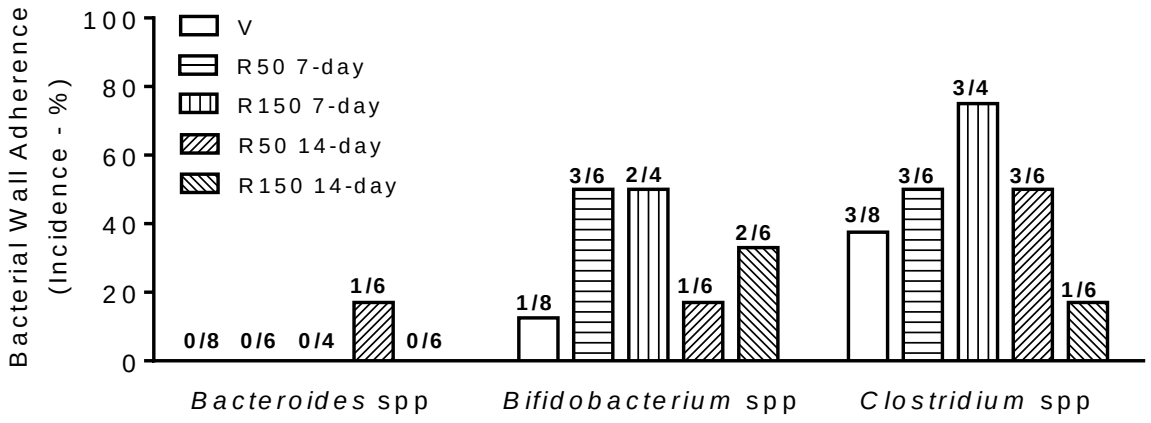

Figure 6. Incidence of bacterial wall adherence. Data represent the percentage of animals showing bacterial wall adherence. Numbers on top of columns represent the number of animals showing bacterial wall adherence over the total number of animals. For the sake of clarity, and since differences were not observed among them, vehicle-treated animals have been merged in a single group (n=8). V: Vehicle; R50: rifaximin at 50 mg/kg; R150: rifaximin at 150 mg/kg.

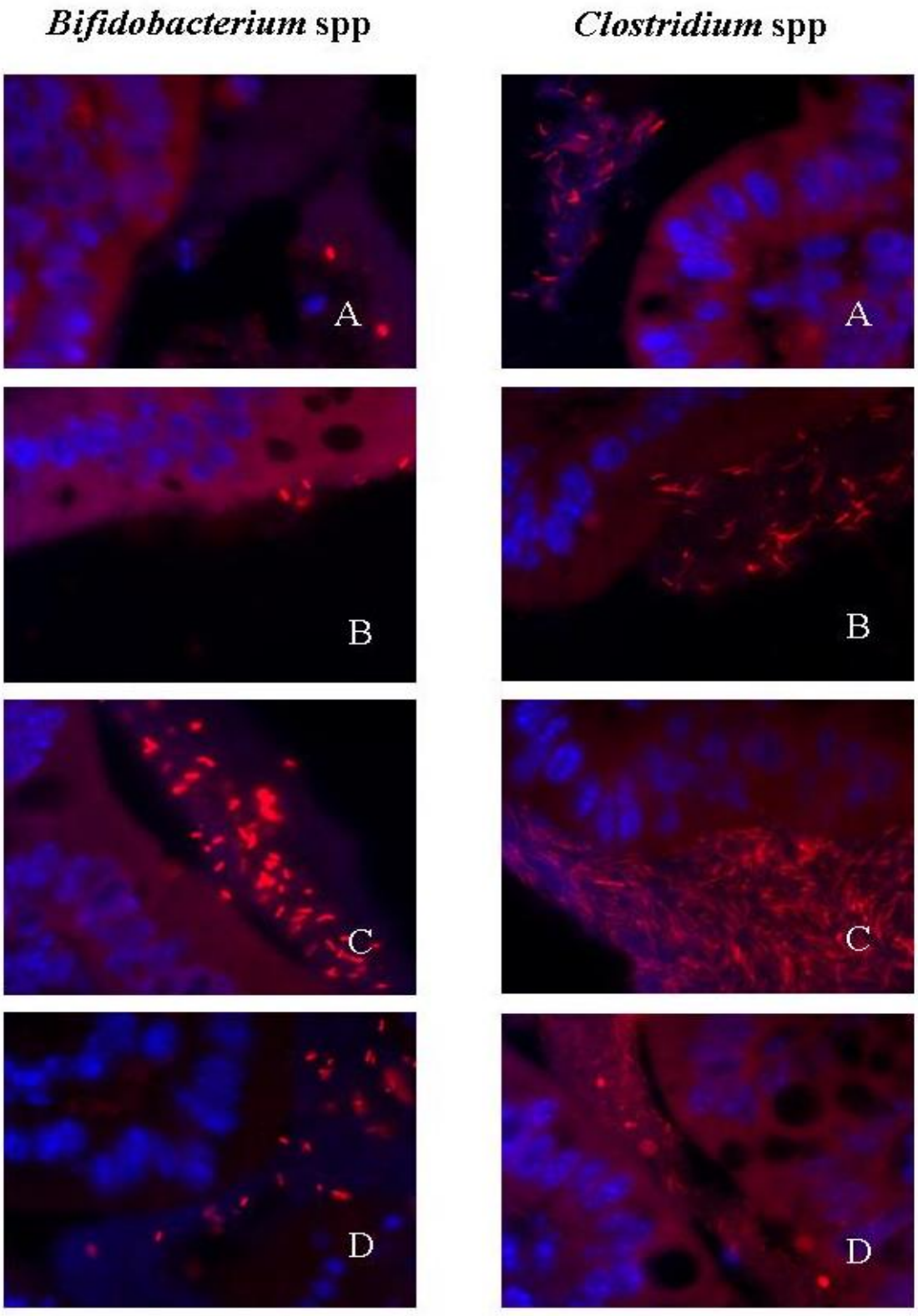

Figure 7. Bacterial wall adherence. Representative images showing bacterial adherence to the colonic epithelium for *Bifidobacterium* spp. and *Clostridium* spp.. A: non-adhered bacteria within the intestinal lumen. B: adhered bacteria in a vehicle-treated mice (7-day), C: adhered bacteria in a rifaximin-treated mice (50 mg/kg, 7-day); and D: adhered bacteria in a rifaximin-treated mice (150 mg/kg, 7-day).

Effects of rifaximin on colonic expression of TLRs

Expression of TLR2, 3, 4, 5 and 7 was detected in all colonic samples. Overall, rifaximin induced minor changes in TLRs expression with only a moderate (2- to 3-fold), but significant, up-regulation observed for TLR3 and 4 for the 50 mg/kg dose during a 7-day period (Fig. 8).

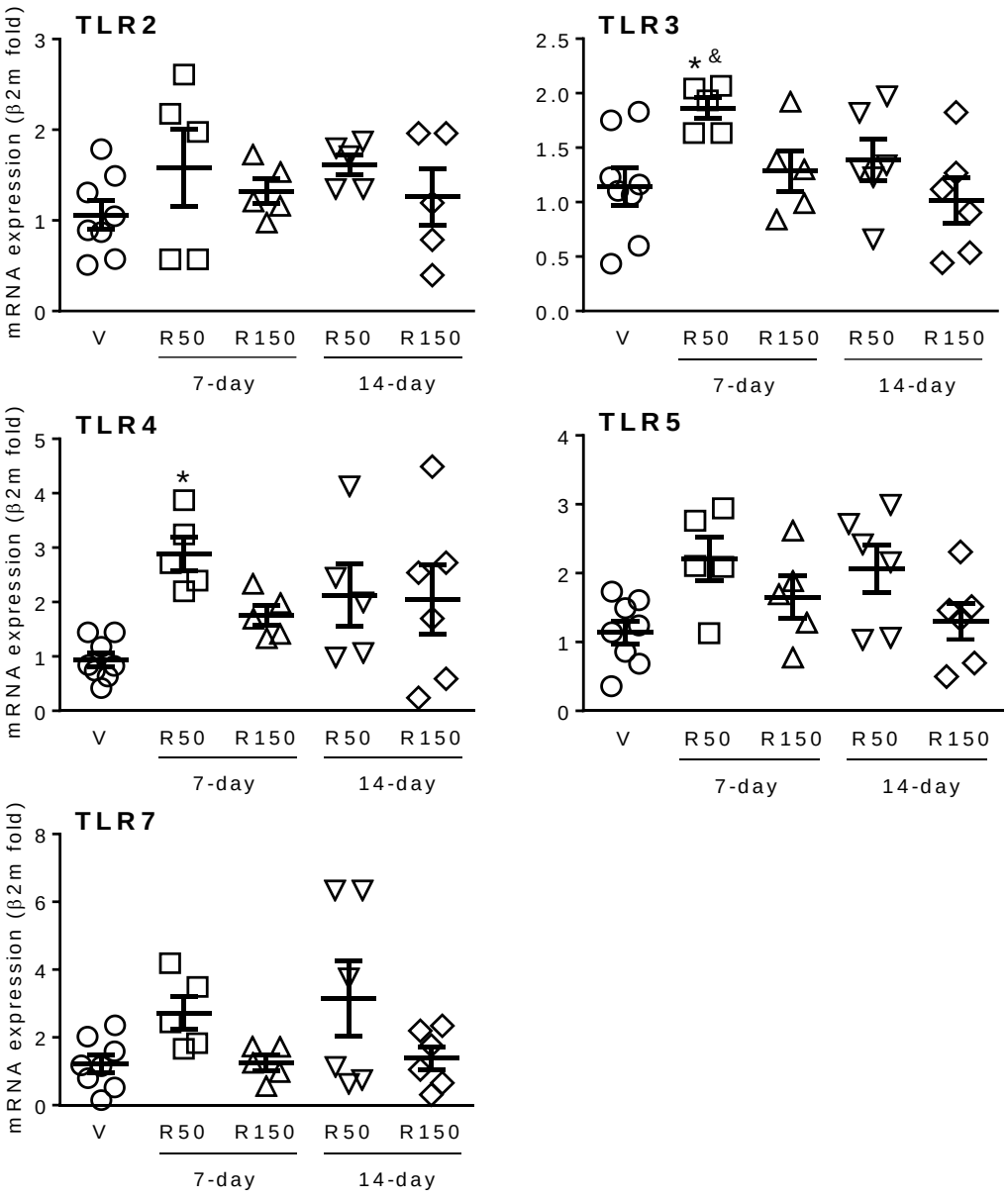

Figure 8. Colonic expression of TLRs. Each point represents an individual animal; the horizontal lines with errors correspond to the mean ± SEM. For the sake of clarity, and since no differences were observed among them, vehicle-treated animals have been merged in a single group (n=8). *: $p<0.05$ vs. V; &: $p<0.05$ vs. R150 14-day. V: Vehicle; R50: rifaximin at 50 mg/kg; R150: rifaximin at 150 mg/kg.

Effects of rifaximin on colonic expression of immune-related markers

Expression of all markers assessed was detected in colonic tissues although in some cases with relatively high variability. Regardless the dose and time of treatment, no changes were observed for pro-inflammatory cytokines (IL-6, IL-18, IFNγ and IL-12p40), anti-inflammatory cytokines (IL-10) or antimicrobial peptides (Defα24, RELMβ and RegIIIγ) (Fig. 9).

Discussion

In the present study, we assessed the effects of the non-absorbable, wide spectrum antibiotic rifaximin on GCM in healthy mice. Overall, rifaximin did not alter the homeostatic state of the gastrointestinal tract expected in healthy animals. Results obtained show that rifaximin did not lead to major alterations in the microbial ecosystem of the gastrointestinal tract. Furthermore, rifaximin did not affect the local (colonic) expression of different immune-related markers related to host-bacterial interactions.

Even though rifaximin has broad-spectrum activity against aerobic and anaerobic gram-positive and gram-negative bacteria, we show that during rifaximin treatment the GCM of standard, healthy mice was essentially not affected. Total bacterial counts in vehicle-treated mice was in the range previously described[23,31] and was not altered after a 7-day or a 14-day treatment with the antibiotic. In agreement with this, bacterial biodiversity, as assessed by a T-RFLP analysis, was not affected by rifaximin. This is consistent with previous studies showing no changes in total fecal bacterial counts after rifaximin treatment in patients with intestinal inflammation[2,32] or diarrheal disease[33] or in pre-clinical models[34]. However, these results contrast with data showing reductions in total fecal bacterial counts in rats after a 3-day treatment period at similar doses to those used here[35] or in ileal bacterial load in rats subjected to psychological stress.[17,36] These apparent discrepancies might reflect species-, treatment protocol- or disease state-related differences.

Lack of effects of rifaximin inducing a clear dysbiosis in the present experimental conditions, against its expected antimicrobial effects and compared to other antibiotic treatments[20,24,37] might suggest a lack of efficacy of the treatment. However, the doses tested are in the range of those used in other reports showing biological activity.[17,35,36,38] Moreover, it does not seem related

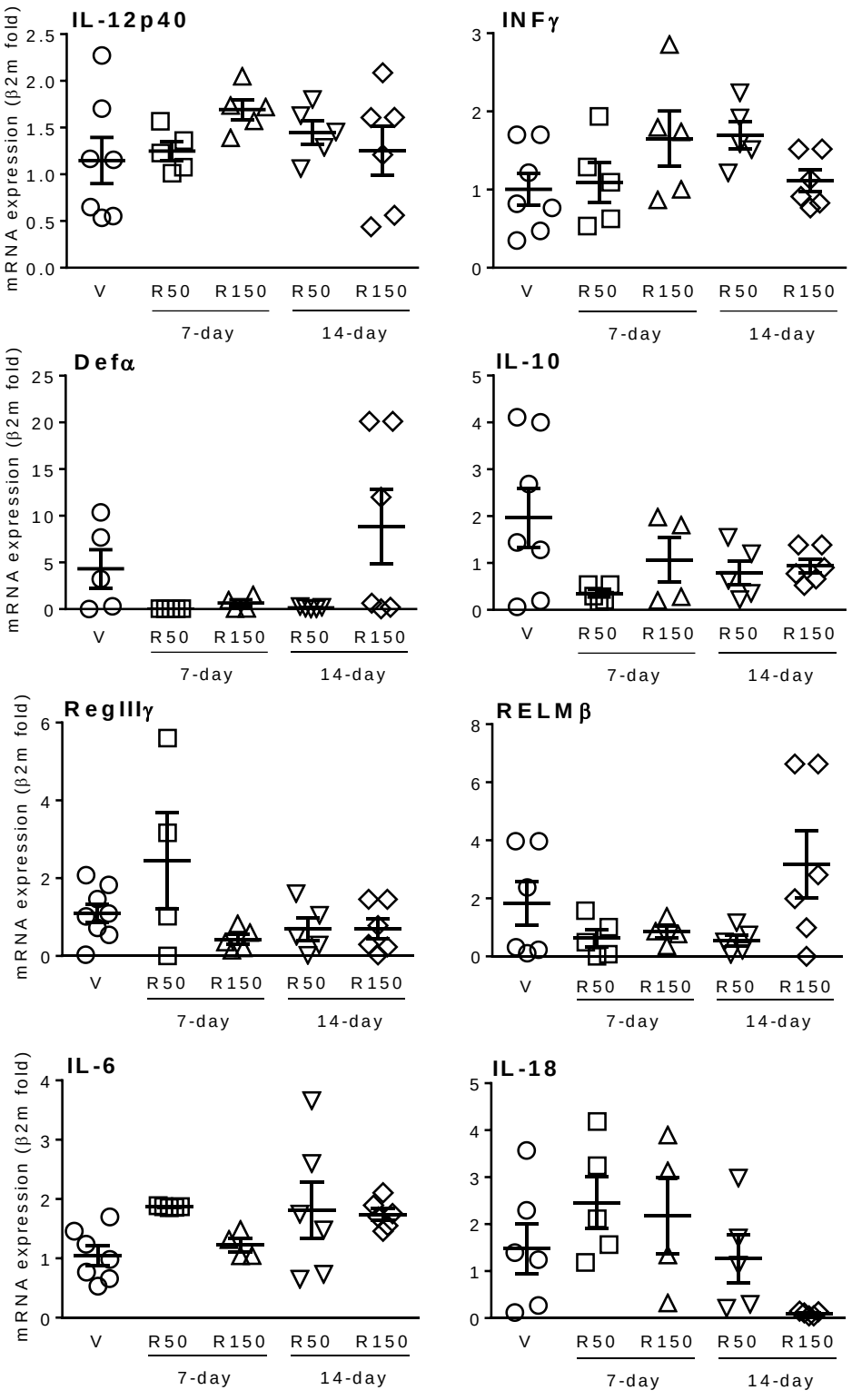

Figure 9. Colonic expression of immune-related markers. Each point represents an individual animal; the horizontal lines with errors correspond to the mean ± SEM. For the sake of clarity, and since no differences were observed among them, vehicle-treated animals have been merged in a single group (n=8). V: Vehicle; R50: rifaximin at 50 mg/kg; R150: rifaximin at 150 mg/kg.

to a loss of activity of the antibiotic since *in vitro* testing using classical microbiological culture procedures showed efficacy against *Staphylococcus aureus* and *Escherichia coli* (data not shown).

Although without overall effects in total bacterial counts, rifaximin has been suggested to modulate the composition of the microbiota.[2,18,32,39,40] To assess this, we determined changes in specific bacterial groups that are recognized as either beneficial (such as lactic acid bacteria like *Lactobacillus/Enterococcus* spp.*, and Bifidobacterium* spp.) or harmful bacteria (such as *Clostridium* spp., Bacteroides spp., and Enterobacteria).[41] The only bacterial group affected by rifaximin was *Bacteroides* spp., which presence was favored by the antibiotic during the 7-day treatment, with a similar tendency observed during the 14-day treatment period. This is in agreement with recent studies in a murine model of ankylosing spondylitis in which rifaximin treatment increased the population of *Bacteroidetes*.[39] However, it contrasts with previous results showing exclusively an increase in *Bifidobacterium* spp. counts after a treatment with rifaximin.[2,32] Again, these contradictory results might be related to differences in the experimental conditions and/or reflect species related-differences (human vs. rodent). Composition of GCM is different in humans and rodents and differs significantly among rodent strains depending upon their breeder and their housing conditions.[21,23] For instance, rifaximin treatment also failed to affect microbiota in mice with a humanized microbiota,[42] further emphasizing the importance of species-related aspects when assessing the microbiota.

The exact mechanisms by which rifaximin improves disease symptoms in inflammatory bowel disease, irritable bowel syndrome or diarrheic disease remain largely unknown.[18] In agreement with the limited effects of rifaximin in intestinal microbiota (either in normal or dysbiotic conditions) evidences suggest the existence of antibiotic-independent effects, likely modulating the local immune environment within the gastrointestinal tract. In this sense, changes in host-bacterial interaction, through the modulation of bacterial wall adherence, represent an attractive alternative mechanism of action, since only epithelium-attached bacteria are able to signal to the host leading to immune-related responses.[18,43–45] In our conditions, bacterial attachment to the colonic epithelium, as assessed by FISH of tissue samples from the colon, was only occasionally observed in control animals. In particular, only Clostridia and Bifidobacteria, considered harmful and beneficial bacteria, respectively, were found attached to the colonic epithelium. Treatment with rifaximin did not affect this pattern, although a slight, and parallel, tendency to increase the incidence of attachment was observed for both bacterial groups during the 7-day treatment; with a progression towards control values for the 14-day treatment groups. The fact

that rifaximin seems to promote the adherence of beneficial commensal bacteria at the same time than pathogenic microorganisms may contribute to its beneficial effects in the treatment of SIBO. In these conditions, the balance between negative and positive signals mediated through the interactions with harmful and beneficial bacteria, respectively, might inhibit dysbiosis-associated negative responses in the host, promoting the restoration of intestinal homeostasis, including a state of normobiosis.

Some evidences suggest also direct effects of rifaximin on intestinal epithelial cells, likely modulating local immune responses. To evaluate this possibility, we also assessed potential changes in the expression of local (colonic) immune-related markers during rifaximin treatment. Overall, no significant changes were observed in the expression of cytokines, either pro- or anti-inflammatory, antimicrobial peptides or TLRs. In fact, only a moderate up-regulation was observed for TLR3 and TLR4 (50 mg, 7-day treatment). Few studies have addressed the effects of rifaximin on immune-related markers in control/healthy conditions. In this respect, no effects on inflammatory mediators after rifaximin treatment were observed in healthy animals treated with the antibiotic.[16,35] In states of altered gut function or chronic systemic inflammatory conditions (implicating also the gastrointestinal tract) both anti-inflammatory activity[39] and no effects on inflammation within the gut[16,42] have been reported for rifaximin. Similarly, Yang et al. (2019)[39] reported only a moderate down-regulation of intestinal TLR4 in a murine model of ankylosing spondylitis. Altogether, additional studies in control conditions (healthy animals) as well as in pathophysiological states involving the gastrointestinal tract are necessary to fully understand the direct, antibacterial-independent, effects of rifaximin on gastrointestinal immune responses.

In our study, rifaximin was tested in standard, healthy animals, so we cannot exclude that the antibiotic might have a more pronounced effects in a disease-state model. This hypothesis warrants further follow-up studies.

As mentioned, several studies showed that antibiotic treatment in normal animals lead to a state dysbiosis concomitant to an immune activation, the induction of intestinal inflammation and the modulation of visceral sensory-related systems.[20,24,37,46] In our case, no evidence of immune activation or colonic alterations consistent with the development of an inflammatory-like state was observed upon macroscopical or microscopical examination of the colon. These differences

are likely due to differences in the antibiotics used and their mechanism of action (including potential antimicrobial-independent effects, as discussed above).

In summary, our results show that rifaximin, even though its antibacterial properties, induces very minor changes in GCM and bacterial wall adherence in normal mice, without changes in local immune-related markers. Although these observations, more noticeable effects of rifaximin on dysbiotic states, vs. a normal GCM, cannot be excluded. Therefore, further studies in dysbiotic animals should be performed to fully assess the effects and mechanism(s) of action of rifaximin within the gastrointestinal tract in order to fully understand the beneficial effects of the antibiotic in functional and inflammatory gastrointestinal disorders.

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Chapter 2

Modulatory Effects of Rifaximin on Inflammation and Gut Commensal Microbiota in the Model of Dextran Sulfate Sodium (DSS)-Induced Colitis in Mice

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Abstract

Background: Rifaximin is a wide-spectrum antibiotic that ameliorates intestinal inflammation in inflammatory bowel disease (IBD), although the mechanism of action remains to be fully elucidated. **Aims:** To characterize the effects of rifaximin in gut commensal microbiota (GCM) and innate immune parameters related to host-bacterial interactions, namely Toll-like receptors (TLRs) and antimicrobial peptides, in a murine model of colitis. **Methods**: C57BL/6NCrl female mice received 3% DSS in the drinking water (5 days). Animals were treated, in a preventive manner, with rifaximin (50 or 150 mg/kg/day, 8 days, PO) or doxycycline (30 mg/kg/day, 8 days, PO). Daily clinical signs, histological colonic inflammation, GCM (16S rRNA gene sequencing), local expression (RT-qPCR) of Toll-like receptors (TLRs) (2, 3, 4, 5 and 7), antimicrobial peptides (DEFα24, RELMβ and RegIIIɣ), inflammatory markers (IL-6, INFɣ, TNFα, IL-1β, RANTES, IL-10) and pregnane X receptors (PXR) were assessed. **Results**: Animals receiving DSS showed clinical signs indicative of the development of colitis. Regardless the dose tested, rifaximin did not affect the clinical course of colitis, while doxycycline, attenuated clinical signs. Similarly, colitis-associated up-regulation of inflammatory markers was not affected by rifaximin, while doxycycline completely prevented this response. Colitis up-regulated RegIIIɣ, while other antimicrobial peptides were unaffected. Rifaximin did not affect colitis-associated RegIIIɣ up-regulation, while doxycycline completely normalized its expression. PXR was down-regulated during colitis, a change not affected by rifaximin but prevented by doxycycline. Regardless the treatment, minor changes in TLRs expression were observed. DSS-induced colitis was associated to a dysbiotic state. Rifaximin–treated animals, with or without colitis, showed similar bacterial composition. Doxycycline-treated-animals showed extensive changes in the microbiota, with similarities between inflamed and non-inflamed conditions. **Conclusions**: In the DSS-induced colitis model rifaximin does not show neither clear anti-inflammatory nor antimicrobial activities. The mechanism mediating the beneficial effects of rifaximin in IBD remains elusive. On the other hand, doxycycline exhibited clear anti-inflammatory activity and modulated in a differential manner GCM, thus warranting further studies exploring potential beneficial effects of doxycycline-induced intestinal dysbiosis.

Key words: colitis, doxycycline, dysbiosis, gut commensal microbiota, host-bacterial interactions, rifaximin, TLRs

Introduction

Inflammatory bowel disease (IBD) is an inflammatory disorder of the gastrointestinal (GI) tract with two distinct manifestations, Crohn's disease (CD) and ulcerative colitis (UC). IBD is characterized by chronic and relapsed inflammation of the gut with unknown etiology.[1] The primary cause of IBD is not completely clear; however, the prevailing view is that it is triggered by multiple factors, including genetic predisposition, environmental factors, gut commensal microbiota (GCM) and disturbances in the innate and adaptive immune responses.[2,3] Growing evidences support the view that IBD results from abnormal immune responses to the GCM.[4–8] A key role of gut microbiota in IBD has long been postulated; however it is not clear whether dysbiosis is truly causative or merely a consequence of the disease.[3,9]

The GI tract represents the most densely colonized organ of the body, with the highest microbial load in the large intestine.[9] The epithelium is in constant contact with both pathogen and beneficial commensal bacteria; therefore, the mucosal immune system has the challenge to act against pathogens while remaining tolerant to commensal microbiota.[10]. These interactions between gut microbiota and the host are known to be mediated through a conserved set of pattern recognition receptors (PRR). Toll-like receptors (TLRs) are among the best characterized PRRs.[11] Pathogenic microorganisms are sensed by TLRs which triggers nuclear factor κβ (NF-κβ) activation and the secretion of pro-inflammatory cytokines.[3] In turn, the interaction between the epithelium and gut microbiota helps the maturation and shaping of immune responses.[12] However, disruption of this delicate equilibrium leads to an increased susceptibility to inflammation.[3]

Taking into account this potential role of microbiota in intestinal inflammation, restoring the normal gut microbiota might be essential for recovering homeostasis after inflammation.[3] In this respect, broad-spectrum antibiotics have shown some therapeutic efficacy in IBD as well as in animal models of the disease.[13] Moreover, immunomodulatory antibiotics might also target the inflammatory response *per se*, thus modifying two of the main components of intestinal inflammation. In this context, the antibiotic rifaximin has shown efficacy for intestinal pathologies implicating both microbial and inflammatory components. Rifaximin is a non-absorbable antibiotic with a broad-spectrum activity against gram-positive and gram-negative micro-organisms.[14] It has been shown to be effective for a variety of clinical uses, with 3 currently approved by the FDA: treatment of traveler's diarrhea, liver disease and irritable bowel

syndrome with diarrhea.[15] The main advantage of rifaximin is that it is virtually unabsorbable, which minimizes systemic exposure and adverse events in all patient populations.[16] Clinical trials have also indicated the potential role for rifaximin in IBD. However, the role of rifaximin in IBD therapy and its mechanisms of action are not fully understood[14,17] although both microbial dependent and independent mechanisms have been suggested.[18] Some studies indicate that rifaximin represents a non-traditional antibiotic with limited antimicrobial activity, but able to act as a 'eubiotic', restoring normal GCM. Indeed, in IBD patients, despite its potent *in vitro* antibiotic activity, rifaximin administration does not appear to alter the overall composition of the gut microbiota, but can change the relationship between different species modulating their abundance.[19,20] Additionally, rifaximin might exert immune-modulatory effects. In humans, rifaximin acts also as a selective pregnane X receptor (PXR) agonist and recent evidences suggest that the antibiotic induces the intestinal expression of PXRs and generates immune-modulatory responses through it.[14,21]

Based on this, the present study aims to describe the effects of rifaximin in intestinal inflammation using the model of dextran sulfate sodium (DSS)-induced colitis in mice. Both, potential immunomodulatory and microbial effects of rifaximin were determined. The immunomodulatory activity was characterized assessing changes in the clinical course of the disease, macroscopic and microscopic colonic alterations and changes in the local expression on immune-related markers, including pro- and anti-inflammatory cytokines as well as host-bacterial interactions markers (TLRs and antimicrobial peptides). Modulatory effects of the antibiotic on the microbiota were assessed by 16S rRNA gene sequencing. For comparative purposes, we also assessed, in equal experimental conditions, the effects of the antibiotic doxycycline on the same parameters. Doxycycline is an immunomodulatory tetracycline with demonstrated anti-inflammatory and antimicrobial effects on different models of intestinal inflammation[22–25], thus serving as a positive control.

Materials and Methods

Animals

Female C57BL/6NCrl mice (n=88), 6 week-old upon arrival, were obtained from Charles River Laboratories (Lyon, France). All animals were group-housed (2-6 animals per cage) under

controlled conditions (temperature: 20-22ºC; 12:12 h light-dark cycle) and unrestricted access to standard chow and tap water. Mice were allowed to acclimatize to these conditions for a 1-week period prior to any experimentation. Thereafter, mice were randomly divided into 8 experimental groups (Table 1). All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocol 1906) and the Generalitat de Catalunya (protocol 7196).

Table 1. Experimental groups[1]

Experimental group	ID	n	Treatment 1 (experimental days -1–6)	Treatment 2 (experimental days 0–5)
1	VV	11	PBS	Water
2	RV50	5	Rifaximin,50 mg/kg	Water
3	RV150	7	Rifaximin, 150 mg/kg	Water
4	DoV	8	Doxycycline, 30 mg/kg	Water
5	VD	17	PBS	3% DSS in water
6	RD50	9	Rifaximin, 50 mg/kg	3% DSS in water
7	RD150	17	Rifaximin, 150 mg/kg	3% DSS in water
8	DoD	14	Doxycycline, 30 mg/kg	3% DSS in water

[1]: See also Fig. 1 for an overview of the experimental design.

Reagents

Rifaximin [4-Deoxy-4'-methylpyrido(1',2'-1,2)imidazo(5,4-c)rifamycin SV; reference R9904; CAS Number 80621-81-4; Sigma-Aldrich, St. Louis, Missouri, USA] was dissolved, under sonication, in sterile PBS at a final concentration of 50 mg/ml, then aliquoted and frozen (-20ºC) until use. Subsequent dilutions to obtain the desired concentrations were freshly made, on a daily basis, using sterile PBS. Doxycycline (doxycycline hydrochloride hemiethanolate hemihydrate; reference D9891; CAS Number 24390-14-5; Sigma-Aldrich) powder was stored at 4 – 8ºC and dilutions were prepared freshly, on a daily basis, using sterile PBS. Dextran sulfate sodium (DSS) (40-60 kD; reference DB001; CAS Number 9011-18-1; TdB Consultancy AB, Uppsala, Sweden) was stored dry in a well-sealed container at room temperature. Fresh DSS

solutions were prepared daily during the 5-day treatment period. PBS or normal tap water, as appropriate, were used as the control treatment.

Experimental protocols

Animals were dosed by oral gavage (0.2 ml/mice) with either rifaximin, doxycycline or vehicle (sterile PBS). All treatments were performed in the morning, between 8:00 and 10:00 AM; during 7 consecutive days (Fig. 1). In addition, starting the second day of treatment, some animals received 3% DSS in the drinking water (wt/v) during a 5-day period. All animals were weighed daily at the time of dosing. Animals were euthanized on experimental day 7, 24 h after the last treatment, for the assessment of inflammation and samples collection (Fig. 1).

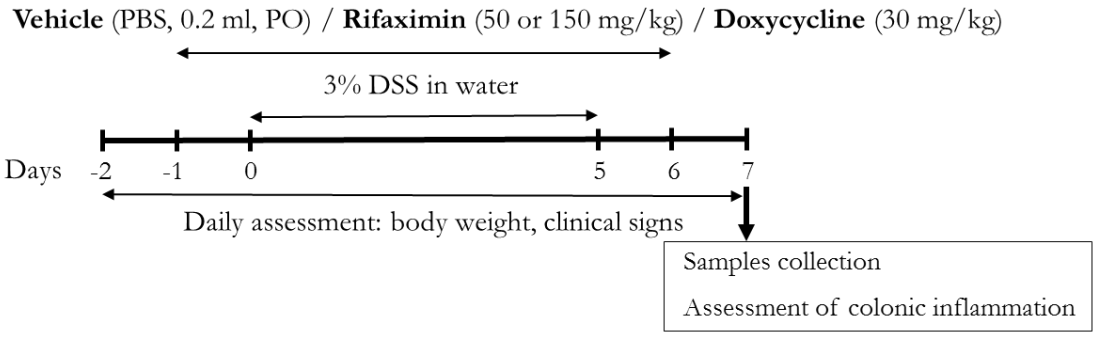


Figure 1. Overview of the experimental protocols.

Clinical and macroscopic assessment of inflammation

Clinical assessment of inflammation included daily monitoring of body weight, appearance of feces and general health condition, according to previously published scores.[26] Separate scores (0–3) were assigned for fecal consistency and health condition; where 0 indicates normal fecal content/healthy condition, 1 indicates loose fecal content/signs of hunch posture and/or piloerection, 2 indicates watery diarrhea/severe hunch posture and piloerection and 3 indicates blood in feces/prostrated and dehydrated.

At necropsy, colonic length and weight were assessed and its macroscopic appearance scored, in a blind manner, according to previously published scores.[26,27] Briefly, inflammatory signs (including inflammatory score: edema -0–3-, thickness -0–3-, stiffness -0–2- and presence of ulcerations -0–1-), consistency of fecal contents (score 0–3) and presence of visible fecal blood (score 0–3) were assessed, resulting in a maximum total score of 15.

Samples collection

24 h after the last treatment animals were deeply anesthetized with isoflurane (Isoflo, Esteve, Barcelona, Spain) and euthanized by exsanguination through intracardiac puncture, followed by cervical dislocation. A laparotomy was performed, the cecum and colon were dissected and fecal samples from the ceco-colonic region obtained and frozen immediately in liquid nitrogen. Thereafter, the cecum, colon, liver, spleen, thymus and adrenal glands were dissected and weighed. Tissue samples covering the middle region of the colon were fixed overnight in 4% paraformaldehyde (about 3 cm) or frozen immediately in liquid nitrogen (about 1.5 cm). After paraformaldehyde fixation, tissues were paraffin embedded and 5 μm-tick sections obtained for hematoxylin-eosin staining. Frozen samples (ceco-colonic fecal content and colonic tissue) were stored at –80 ºC until analysis.

Histological studies

Paraffin-embedded colonic sections (5 μm thickness) were stained with hematoxylin-eosin following standard histological procedures. Coded sections were observed with a light microscope (Zeiss AxioVision Release 4.8.1 software, Germany) and the epithelial structure, the presence of ulcerations and the inflammatory infiltrate were assessed. A histopathological score (ranging from 0, normal, to 12, maximal alterations) was assigned to each sample, according to previously published scores.[28] Specifically, parameters scored included: epithelial structure (0: normal; 1: mild alterations of the villi; 2: focal villi destruction and/or fusion; 3: generalized villi destruction and/or fusion), crypts (0:normal; 1: mild alterations of the crypts; 2: local destruction of the crypts; 3: generalized destruction of the crypts), edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria) and inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3;

severe generalized infiltrate). All samples were evaluated in a blinded fashion by two separate investigators (MF and VM) and the mean of the two scores was assigned to each animal.

Colonic gene expression using quantitative real-time PCR

Total RNA was extracted from colonic tissue samples using Tri reagent with Ribopure Kit (Ambion/Applied biosystems, Foster City, CA, USA). RNA samples were converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA concentration measured using NanoDrop (ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) and all the samples were diluted at 100 ng/µl with DEPC-Treated water (Ambion/ Applied biosystems, Foster City, California, USA). TaqMan gene expression assays for interleukin 6 (IL-6) (Mm00446190_m1), interleukin 1 beta (IL-1β) (Mm00434228_m1), interferon gamma (INFɣ) (Mm01168134_m1), tumor necrosis factor (TNFα) (Mm00443258_m1), regulated upon activation, normal T cell expressed and secreted (chemokine (C-C motif) ligand 5) (RANTES (CCL5)) (Mm01302427_m1), interleukin-10 (IL-10) (Mm00439614_m1), defensin alpha 24 (Defα24) (Mm04205950_gH), resistin-like molecule beta (RELMβ) (Mm00445845_m1), regenerating islet-derived protein 3 gamma (RegIIIɣ) (Mm00441127_m1), Toll-Like Receptors 2 (TLR2) (Mm00442346_m), 3 (TLR3) (Mm01207404_m1), 4 (TLR4) (Mm00445273_m1), 5 (TLR5) (Mm00546288_s1) and 7 (TLR7) (Mm00446590_m1) and pregnane X receptor (PXR) (Mm01344139_m1) were used (Applied Biosystems). All samples, as well as the negative controls, were assayed in triplicates. B-2-microglobulin (β2m) (Mm00437762_m1) was used as endogenous control. The PCR reaction mixture was incubated on the Bio-Rad CFX384 (Bio-Rad Laboratories). Bio-Rad CFX Manager 3.1 software was used to obtain the cycle threshold for each sample. All data was analyzed with the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the vehicle groups serving as calibrator.

Fecal DNA extraction and 16S rRNA gene amplification

In order to assess microbiota composition, ceco-colonic fecal samples collected at necropsy were used. DNA was extracted using a QIAamp® DNA Stool Mini kit (QIAGEN) with a previous step of mechanical disruption to improve cell lysis.[29] Briefly, cells were suspended in 1.4 mL of ASL buffer and 500 µL of 0.1 mm glass beads and tubes were vortex at maximum

speed for 5 min prior to the initial incubation for heat and chemical lysis at 95 °C for 7 min. Subsequent steps of the DNA extraction followed the QIAamp kit protocol.

The V3-V4 region of the 16S rRNA gene was amplified and sequenced using the MiSeq platform from Illumina, as described in the manual for "16S Metagenomic Sequencing Library Preparation" of the MiSeq platform (Illumina Inc., San Diego, USA). Briefly, for each sample, a 25 μL reaction was prepared containing 12.5 ng of DNA, 12.5 μL 2x KAPA HiFi Hot Start Mix, and 0.2 mM of primers. Water was added to complete the volume of the reaction. Cycling conditions were 95 °C for 3 min, and 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final elongation cycle at 72 °C for 5 min. The amplification was confirmed through electrophoresis by loading 4 μL of the PCR reaction on a 1.6% agarose gel. Subsequently, the PCR product was purified with the AMPure XP beads as described in the Illumina protocol. Next, a limited-cycle PCR reaction was performed to amplify the DNA and add index sequences on both ends of the DNA, thus enabling dual-indexed sequencing of pooled libraries. Index PCR consisted of a 50 μL reaction containing 5 μL of the DNA obtained from the previous PCR, 25 μL of 2x KAPA HiFi Hot Start Mix, and 5 μL of forward and reverse indexed primers. Temperature conditions were the same as for the first reaction, but the number of cycles was reduced to 8. The obtained PCR product was purified with the AMPure XP beads following the manufacture's protocol. An equal amount of the purified DNA was taken from each sample for pooling. Each pool of samples was sequenced following Illumina recommendations.

16S rRNA gene sequencing analysis

Sequences were processed using Mothur v1.35[30] as previously described[29], with some modifications. Initial trimming by quality was performed on paired ends of sequences before joining them into a single read. Parameters used for trimming included elimination of sequences shorter than 200 bp or that contained homopolymers longer than 8 bp or undetermined bases. Using the base quality scores, which range from 0 to 40 (0 being ambiguous base), sequences were trimmed using a sliding-window technique, such that the minimum mean quality score over a window of 50 bases never dropped below 20. Sequences were trimmed from the 3' end until this criterion was met. Sequences were aligned to the 16S rRNA gene using as a template the SILVA reference alignment. Potential chimeric sequences were removed using Uchime algorithm. To minimize the effect of sequencing errors in overestimating microbial diversity[31], rare abundance sequences that differ in up to four nucleotides from a high abundant sequence

were merged to the high abundant sequence using the pre.cluster option in Mothur. An average of 90361 sequences was obtained per sample. Since different number of sequences per sample could lead to a different diversity (i.e. more Operational Taxonomic Units -OTUs- could be obtained in those samples with higher coverage), in order to compare the diversity of different fecal samples, we rarefied all samples to the number of sequences obtained in the sample with the lowest number of sequences (55211). In other words, 55211 sequences were randomly selected from each sample for subsequent analysis: taxonomic characterization and OTUs identification. Sequences with distance-based similarity of 97% or higher were grouped into the same OTU using the VSEARCH abundance based greedy clustering (AGC) method. OTU-based microbial diversity was estimated by calculating the Shannon diversity index. Each sequence was classified using the Bayesian classifier algorithm with the bootstrap cutoff of 60%.[32] In most cases classification could be assigned to the genus level. When it was not possible to classify a sequence to a certain taxonomic level, it was assigned as "unclassified" followed by the upper taxonomic level.

Principal coordinate of analysis

In order to compare the overall microbiota similarity between different fecal samples, we calculated for every pair of samples the Bray-Curtis distance with Mothur, using the relative abundance of each OTU identified in each sample. Principal Coordinate of Analysis were performed on the resulting Bray-curtis distance matrix with Mothur.

Statistical analysis

The PERMANOVA test was applied on the Bray-Curtis distance matrix to detect overall changes in the community composition among different groups of samples using the vegan library from the R package (adonis function). In order to determine statistically significant differences in the relative abundance of different taxa and OTUs among experimental groups, the non-parametric Wilcoxon test was applied using wilcox.test function in the "stats" R package. Taxa and OTUs with less than 10 counts in both groups under comparison were not included in the analysis. To adjust for multiple hypothesis testing, we used the FDR approach by Benjamini and Hochberg[33] implemented in the stats package. Taxa with a $p<0.05$ and $FDR<0.1$ were considered statistically significant. Heatmap at genus and OTU level was

performed using GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA). Hierarchical clustering was performed in R with gplots and stats packages using the hclust function. Correlation tests between microbiota abundances and inflammation-related scores and gene expression of immune-related markers were performed using cor.test function ("stats" R package) with Spearman's test implemented in-house R-scripts.

Data from body weight, fecal score, colonic inflammatory scores and relative gene expression are expressed as mean ± SEM. Statistical analysis was performed using SigmaPlot version 12.0 (Systat Software, Inc., San Jose California USA). Data were analyzed by two-way analysis of variance (ANOVA), followed, when necessary, by a Holm-Sidak multiple comparisons test. Correlations between body weight changes, inflammatory scores, inflammatory markers, antimicrobial peptides and TLRs were performed with Spearman's test. Data were considered statistically significant when $p<0.05$.

Results

Rifaximin did not affect the clinical course of DSS-induced colitis in mice

All animals, regardless the experimental group considered, had a similar body weight at the beginning of the study. Animals receiving normal water showed a time-related increase in body weight throughout the experimental period, regardless the treatment applied (Fig. 2A). From day 4, animals from the vehicle-DSS group presented a linear decrease in body weight. A similar profile was observed in inflamed animals treated with rifaximin, although body weight loss at the end of the experiment (day 7) was significantly higher when compared with the vehicle-DSS group (Fig. 2A). Treatment with doxycycline significantly attenuated body weight loss in inflamed animals.

No clinical signs were observed in vehicle-vehicle-treated animals or in animals receiving either rifaximin or doxycycline without DSS exposure (Fig. 2B). DSS-treated groups, regardless the pre-treatment received, showed a time-related increase in the incidence of clinical signs, reaching the higher values by the end of the DSS exposure period. Clinical scores were similar across DSS-treated animals (Fig. 2B).

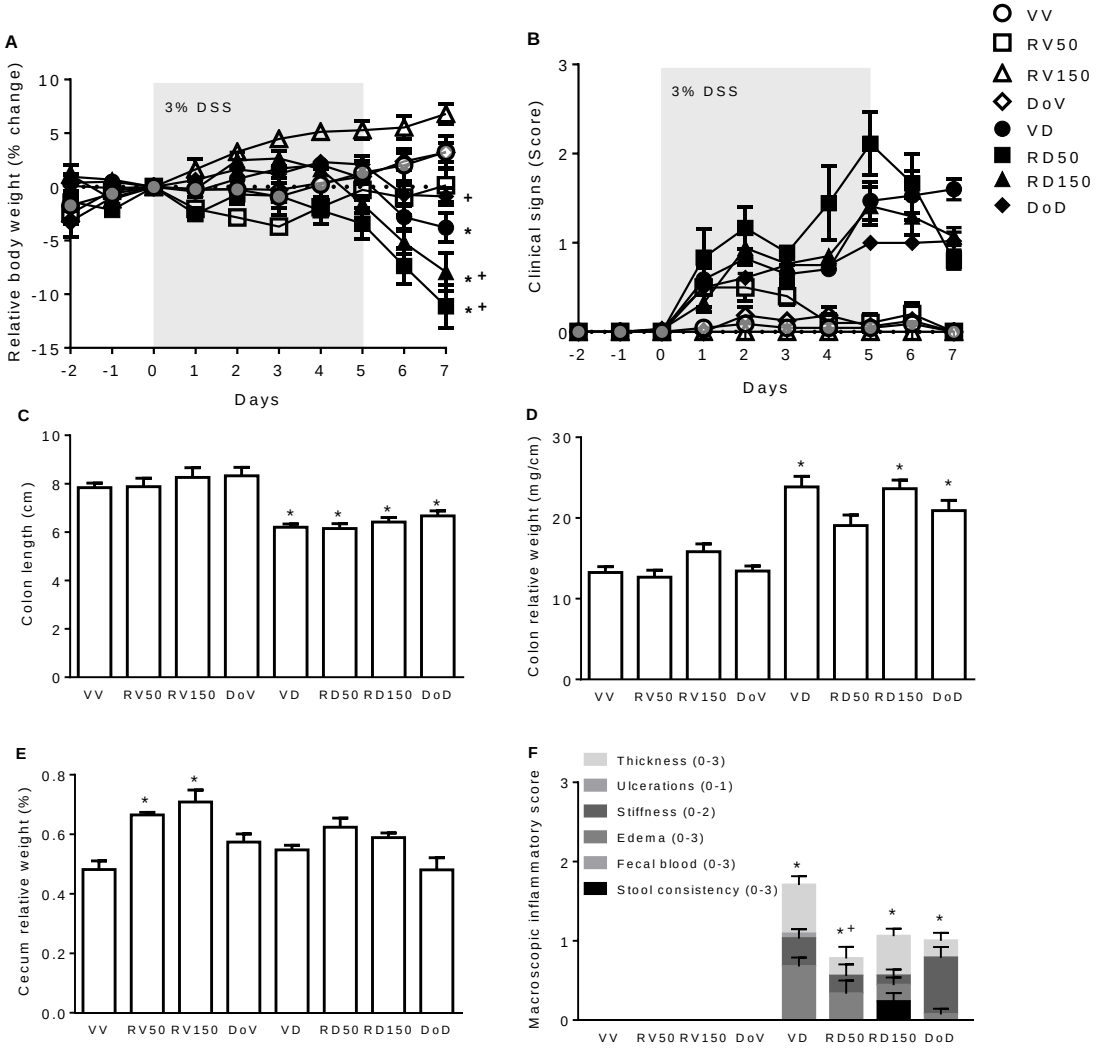


Figure 2. Clinical signs and macroscopic colonic alterations associated to the exposure to 3% DSS. (A) Changes in relative body weight. (B) Clinical signs. (C) Changes in colon length. (D) Colon relative weight. (E) Relative weight of the colon. (F) Macroscopic inflammatory scores. Data represent mean ± SEM (n= 5–17). The shadowed area in A and B represents the 5-day period of 3% DSS exposure. *: $p<0.05$ vs. respective vehicle group. +: $p<0.05$ vs. VD group. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

At necropsy, examination of the colon showed a significant shortening, in a similar degree in all DSS-treated groups (Fig. 2C), and an increase in colonic relative weight (Fig. 2D). In DSS-treated animals, regardless the group considered, macroscopic inflammatory scores were increased when compared to healthy mice (all $p<0.05$); but were attenuated in antibiotic-treated animals, although statistical significance was only achieved for rifaximin at the dose of 50 mg/kg ($p<0.05$ vs. vehicle-DSS group; Fig. 2F). Treatment with rifaximin resulted also in an increase

of the relative weight of the cecum in healthy animals, an effect not observed during colitis (Fig. 2E). As it relates to other organs (liver, spleen, adrenals and thymus), no consistent treatment-related changes in relative weight were observed (data not shown).

Rifaximin did not affect colonic histopathological alterations associated to DSS-induced colitis in mice

Animals receiving normal water, regardless the experimental group considered, showed no microscopic signs of intestinal inflammation, with essentially normal histological features (Fig. 3). On the other hand, histological scores were significantly increased in DSS-treated animals. Histological evaluation of tissue sections from vehicle-DSS-treated group showed epithelial and crypt irregular architecture, presence of edema and moderate infiltration of inflammatory cells. Treatment with rifaximin did not affect these histological alterations. Doxycycline showed a small, but non-significant, attenuation of the histopathological changes observed (Fig. 3).

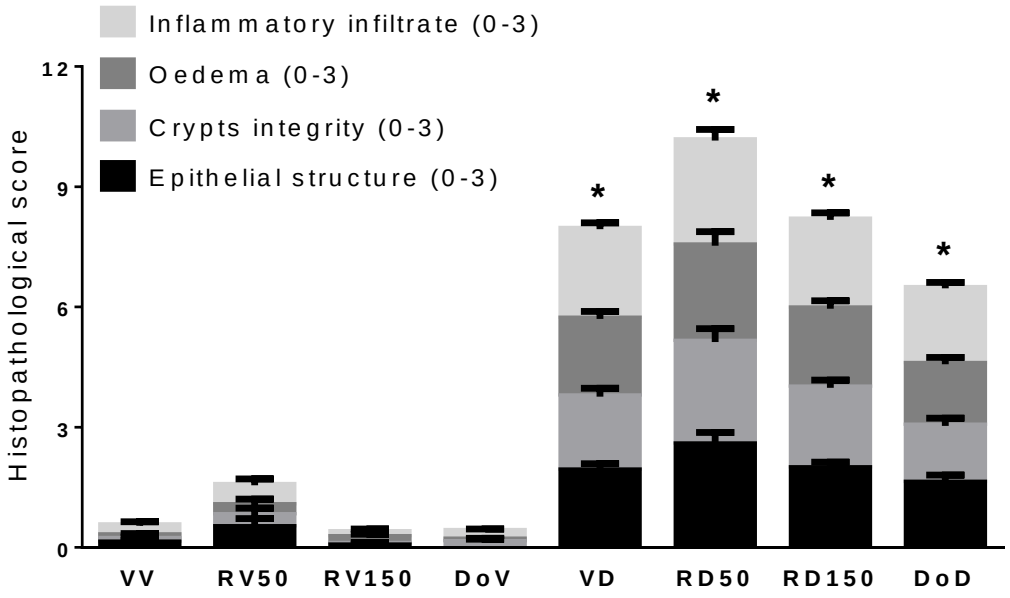


Figure 3. Colonic histopathological inflammatory scores at the time of necropsy. See methods for details about quantification scores. Data represent mean ± SEM (n= 5 – 17). *: $p<0.05$ vs. non-inflamed animals. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

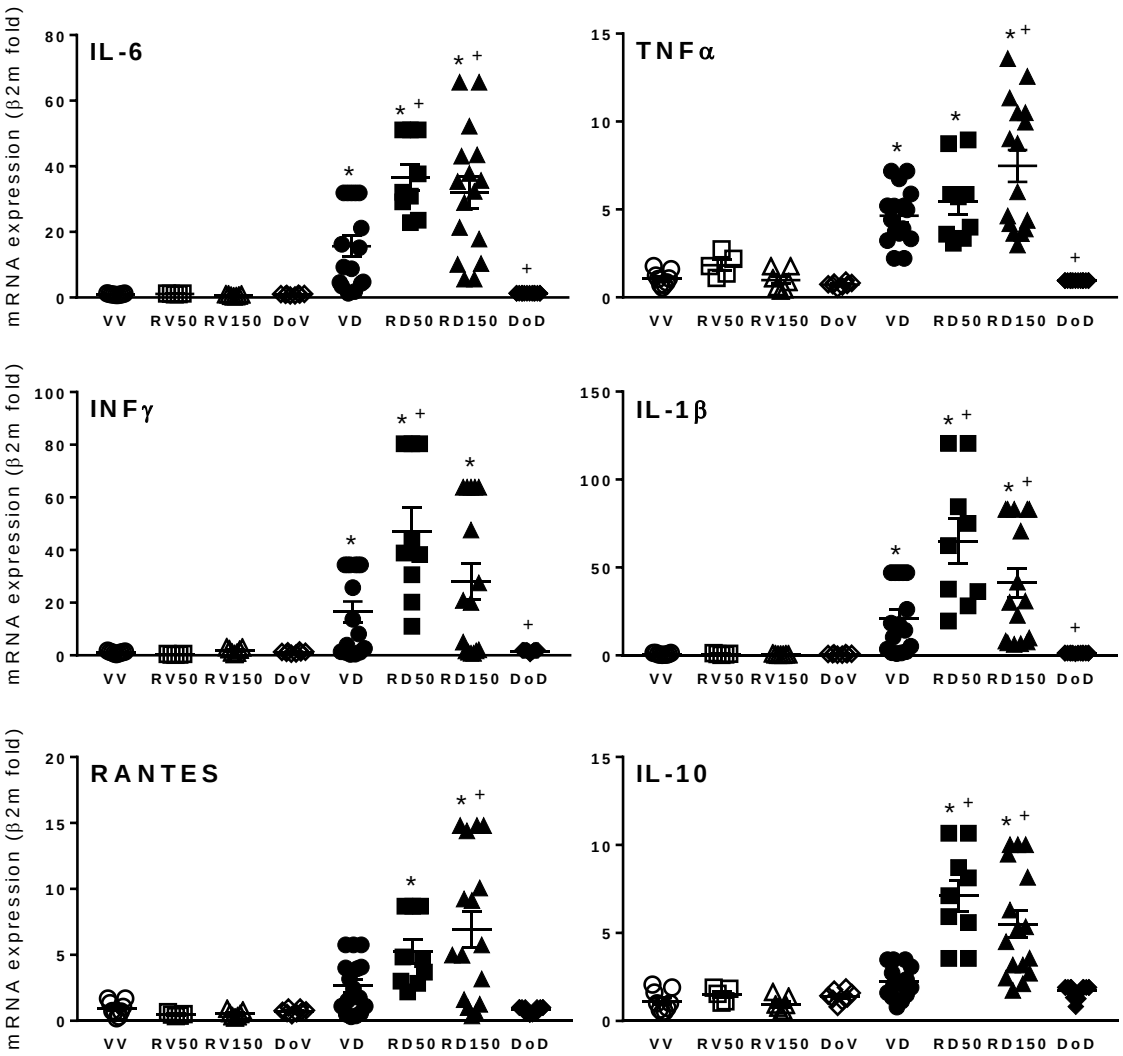


Figure 4. Colonic gene expression of inflammatory markers in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$ vs. respective vehicle group. +: $p<0.05$ vs. VD group.). IL-6, interleukin-6; INFγ, Interferon-gamma; TNFα, Tumor necrosis factor alpha; IL-1β, interleukin-1β; RANTES, regulated upon activation, normal T cell expressed and secreted; IL-10, interleukin-10. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

Colitis-associated up-regulation of inflammatory markers was enhanced by rifaximin

Gene expression of inflammatory markers was detected in all samples analyzed. Animals without colitis, regardless the experimental group considered, showed similar levels of expression of pro-inflammatory cytokines (IL-6, INFγ, TNFα, IL-1β, RANTES) (Fig. 4). In

animals receiving vehicle, exposure to DSS led to a general increase in the expression levels of pro-inflammatory cytokines (Fig. 4). Inflamed animals treated with rifaximin, regardless the dose considered, exhibited a significant, consistent up-regulation in all pro-inflammatory cytokines assessed, compared to non-inflamed animals. Moreover, an enhanced response vs. the vehicle-DSS-treated group was detected in most cases, although no dose-related consistency was observed (Fig. 4). Treatment with doxycycline completely normalized the expression of pro-inflammatory cytokines in DSS-treated animals (Fig. 4).

Expression of the anti-inflammatory cytokine IL-10 was similar in animals without colitis, receiving normal water, regardless the experimental group considered (Fig. 4). In vehicle-treated animals exposed to DSS, a small increase in IL-10 expression was observed (by 2-fold), but statistical significance was not reached. In animals receiving rifaximin, regardless the dose considered, a significant up-regulation in IL-10 gene expression was detected (Fig. 4). Doxycycline did not affect IL-10 expression in DSS-treated animals.

Rifaximin led to minor changes in Toll-like receptors (TLRs) expression

Expression of TLR2, 3, 4, 5 and 7 was detected in all samples analyzed, with relatively low within-group variability (Fig. 5). In non-inflamed animals, expression of TLRs was similar across experimental groups, with only minor changes (by 0.5-fold) observed for TLR3 and TLR4 (both up-regulated at the dose of 50 mg/kg) and TLR5 expression (down-regulated at the dose of 50 and 150 mg/kg) in rifaximin-treated mice (Fig. 5).

Exposure to DSS had minor effects on TLRs expression, with only a minor (by 0.5-fold) down-regulation in the expression of TLR5. In inflamed animals treated with rifaximin, a minor down-regulation (by 0.5-fold) of TLR3, TLR4 and TLR5 was observed. In addition, a minor up-regulation (by 0.5-fold) was observed for TLR7 in inflamed animals treated with rifaximin at 150 mg/kg ($p<0.05$ vs. rifaximin 150 mg/kg-vehicle group). In inflamed animals treated with doxycycline, only a down-regulation of TLR3 and TLR5 (less than 0.5-fold; $p<0.05$ vs. doxycycline-vehicle group) was observed.

Rifaximin had minor effects on antimicrobial peptides

Expression of the antimicrobial peptides RELM-β, Defα24 and RegIIIγ was detected in all samples analyzed. Relatively high inter-individual variability was observed in the expression of

both RELM-β and Defα24, regardless the experimental group considered (Fig. 6). No consistent, treatment-related, changes in the expression of either RELM-β or Defα24 were observed, except for an increase in the RELM-β expression of the non-inflamed animals treated with rifaximin at 50 mg/kg, probably due to the relative large variability observed within this group (Fig. 6).

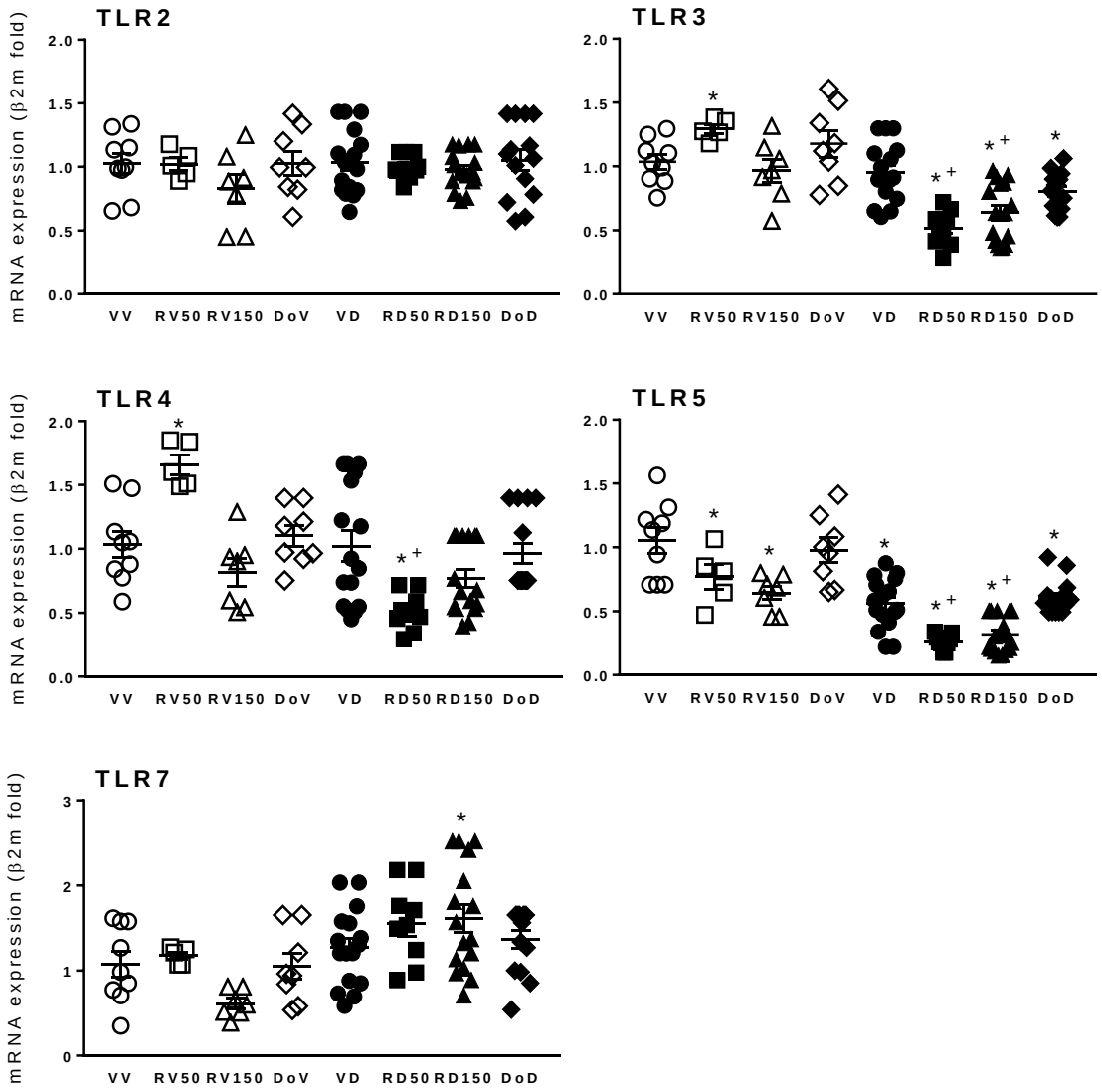


Figure 5. Colonic gene expression of TLRs in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM.*: $p<0.05$ vs. respective vehicle group. +: $p<0.05$ vs. VD group. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

Expression levels of RegIIIγ were similar in all animals without colitis, regardless the experimental group considered. In vehicle-treated animals, colitis was associated to a significant up-regulation of RegIIIγ (by 200-fold; $p<0.05$ vs. vehicle-vehicle group; Fig. 6). Similar up-regulation was observed in rifaximin-treated animals, regardless the dose considered (Fig. 6). Doxycycline completely normalized RegIIIγ expression in inflamed animals.

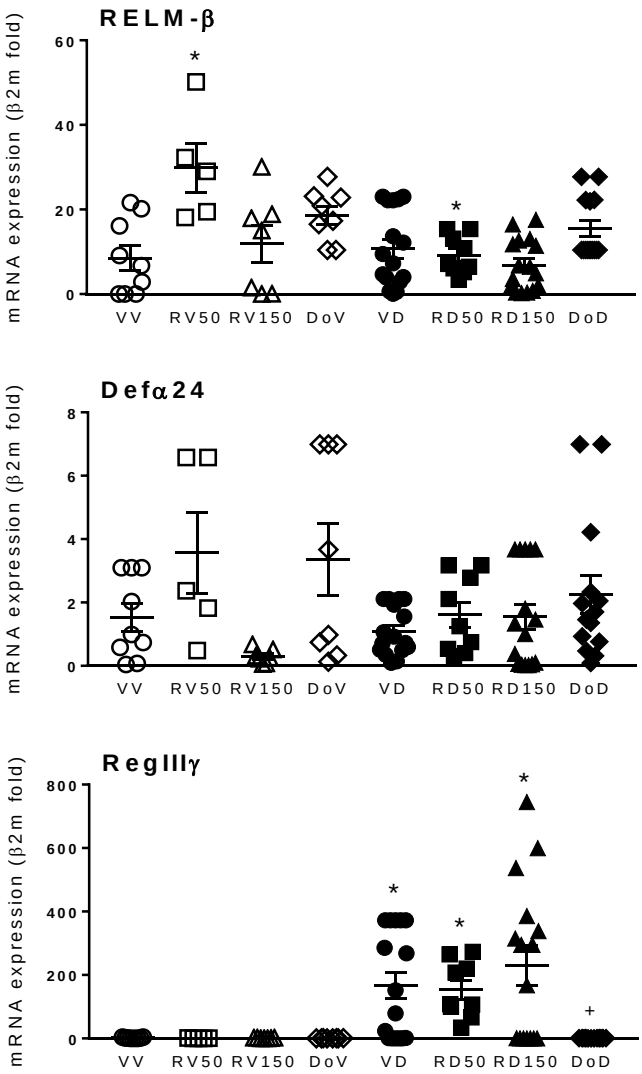


Figure 6. Colonic gene expression of antimicrobial peptides: Resistin-like molecule beta (RELM β), regenerating islet-derived protein 3 gamma (RegIIIγ) and defensin alpha 24 (Defα24). Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$ vs. respective vehicle group. +: $p<0.05$ vs. VD group. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

Intercorrelation between clinical indices of inflammation and molecular markers

A correlation analysis of relevant parameters that presented significant variations during colitis (body weight changes, immune-related markers, inflammatory scores, expression of the antimicrobial peptide RegIIIɣ and expression of TLRs) was performed (Fig. 7). Overall, inflammatory markers positively correlated among them, with RegIIIɣ expression, and with the inflammatory scores; while negatively correlated with TLR3 and TLR5 expression. Body weight correlated negatively with inflammatory scores, immune-related markers and RegIIIɣ, and positively with TLR3 and TLR5 expression. TLRs correlated positively amongst them (Fig. 7).

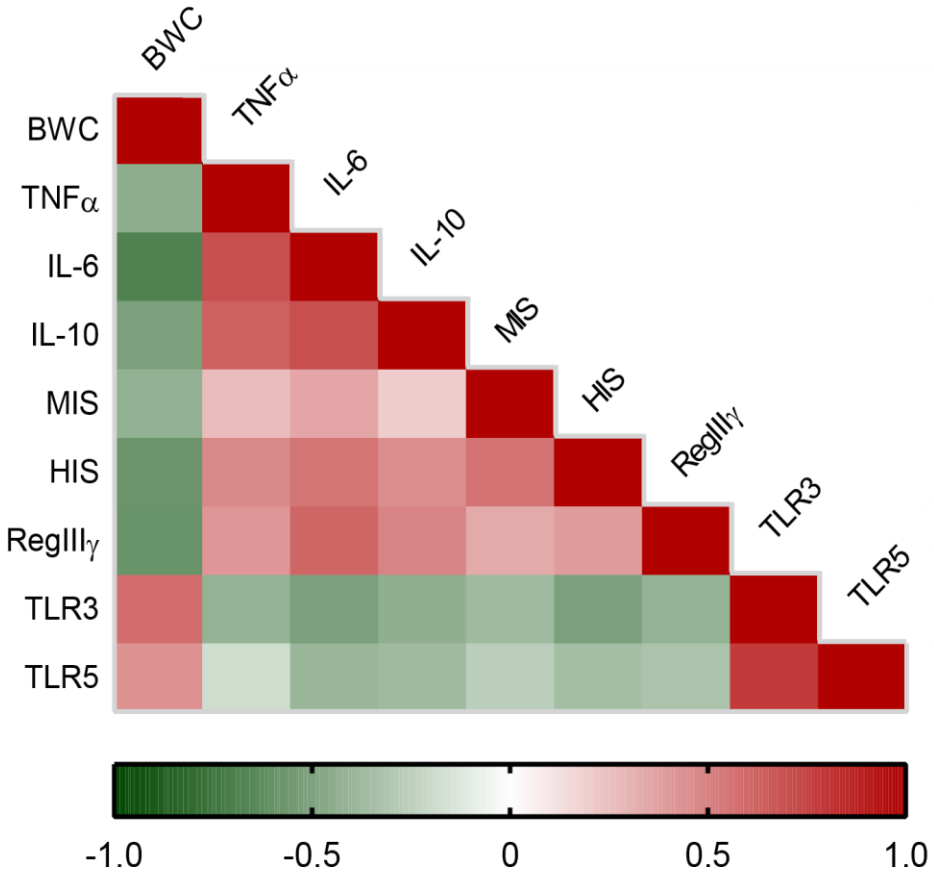


Figure 7. Correlation among body weight changes, inflammatory scores, inflammatory markers, antimicrobial peptides and TLRs during DSS-induced colitis. Correlation analysis was performed by a Pearson correlation test. All data shown correspond to statistically significant correlations ($p<0.05$). Positive or negative correlation is denoted by the color, according to the scale included in the figure. BWC, body weight change; TNFα, Tumor necrosis factor alpha; IL-6, interleukin-6; IL-10, interleukin-10; MIS, macroscopic inflammatory score; HIS, histopathological inflammatory score; RegIIIɣ, regenerating islet-derived protein 3 gamma; TLR, Toll-like receptor.

Effects of rifaximin on PXR expression

PXR expression was detected in all samples analyzed. In animals without colitis, receiving normal water, the expression of PXR was similar across experimental groups. In vehicle-treated animals, DSS exposure had a tendency to down-regulate PXR expression, however large variability was observed among animals (with 9, out of 17, animals showing a clear down-regulation) and statistical significance was not reached (Fig. 8). DSS-treated animals receiving rifaximin, PXR showed a significant down regulation (by 50% vs. vehicle-vehicle or respective rifaximin-treated control group; $p<0.05$ in all cases; Fig. 8). Doxycycline did not affect PXR expression during colitis.

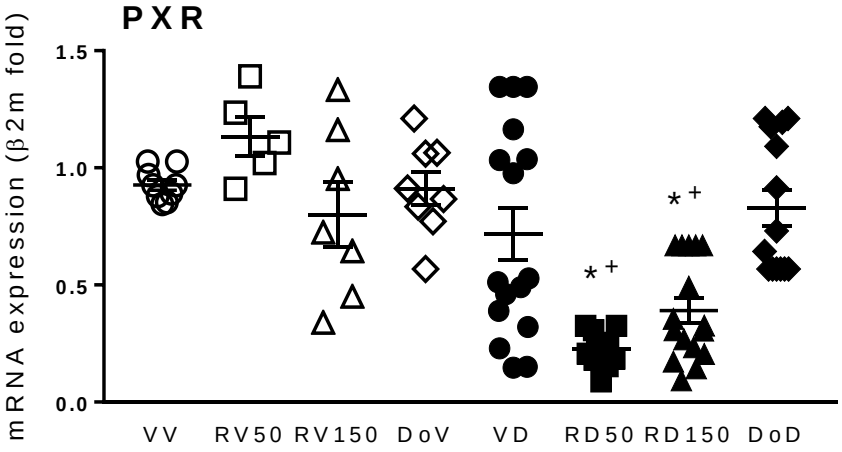


Figure 8. Colonic gene expression of pregnane X receptor (PXR) in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$ vs. non-inflamed animals. +: $p<0.05$ vs. VD group. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

Colitis and rifaximin alter the biodiversity of the ceco-colonic microbiota: Analysis of alpha and beta diversity

Microbial alpha diversity was analyzed by calculating the Shannon and the Sobs indexes of biodiversity. In non-inflamed animals, Shannon and Sobs biodiversity indexes were similar across experimental groups; with no significant effects associated to the treatment with either rifaximin or doxycycline (Fig. 9). Similarly, alpha diversity was maintained after the induction of

colitis, regardless the experimental group considered; with only a small, but significant, reduction observed in doxycycline-treated animals when compared with vehicle-treated inflamed animals (Fig. 9).

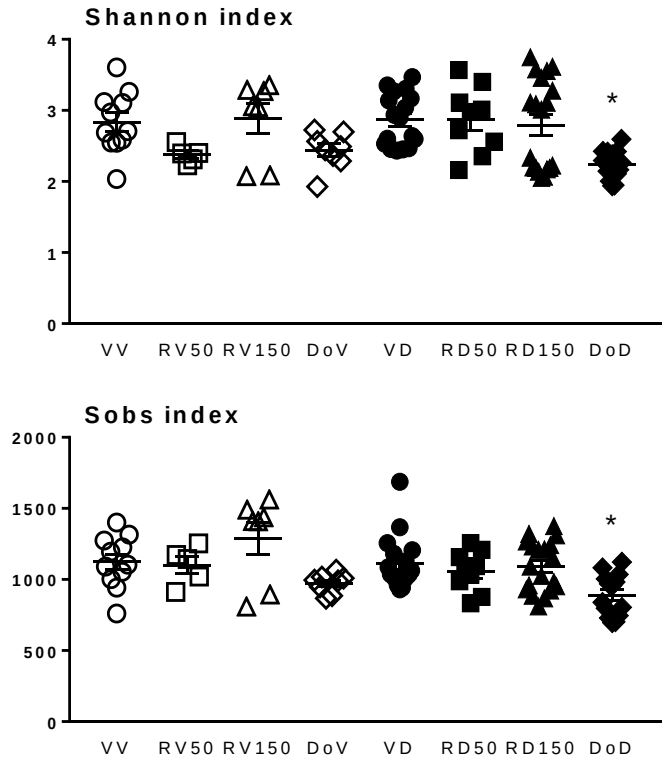


Figure 9. Alpha diversity (within community richness): Shannon and Sobs diversity indices of the ceco-colonic microbiota in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$ vs. VD group. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

Beta (between samples) diversity was assessed through a principal coordinate analysis (PCoA), which clusters communities according to their measure of dissimilarity. The principal coordinate analysis (PC1 and PC2, PC2 and PC3) separated all experimental animals in six different clusters (Figs. 10A-B). A clear separation was observed between vehicle- or rifaximin-treated animals (clusters I, III, IV and VI) from those treated with doxycycline (Clusters II and V). Clusters I, III and VI included all animals from the vehicle-vehicle group and rifaximin-treated groups with or without colitis, with significant overlapping in the distribution of the animals included in

these groups. On the other hand, cluster IV included only vehicle-treated animals with colitis. As mentioned, clusters II and V included all doxycycline-treated animals, with overlapping of animals with and without colitis.

The same clusters of communities as described for the PCoA were also observed in the hierarchical cluster tree (Fig. 10C).

Colitis and rifaximin alter the ceco-colonic microbiota: Phylogenetic analysis

To determine whether bacterial taxa were affected by the different experimental conditions, the average relative abundance of different bacterial groups was determined in ceco-colonic fecal samples and analyzed according to the phylum, family and genus phylogenetic levels. Similarly, an analysis at the OTUs level, comparable to the species phylogenetic level, was also performed. In non-inflamed, vehicle-treated animals ceco-colonic microbiota was dominated by the phyla Bacteroidetes (48.4%) and Firmicutes (37%). Less abundant phyla (<10%) were Verrucomicrobia, Deferribacteres and Proteobacteria (Figs. 11 and 12). At the family level, ceco-colonic microbiota was dominated by the families Porphyromonadaceae (39.9%) and Lachnospiraceae (24.1%), followed by Verrucomicrobiaceae, Ruminococcaceae, unclassified_Clostridiales, Deferribacteraceae, Rikenellaceae and Bacteroidaceae (<10% of abundance) (Fig. 13 and 14).

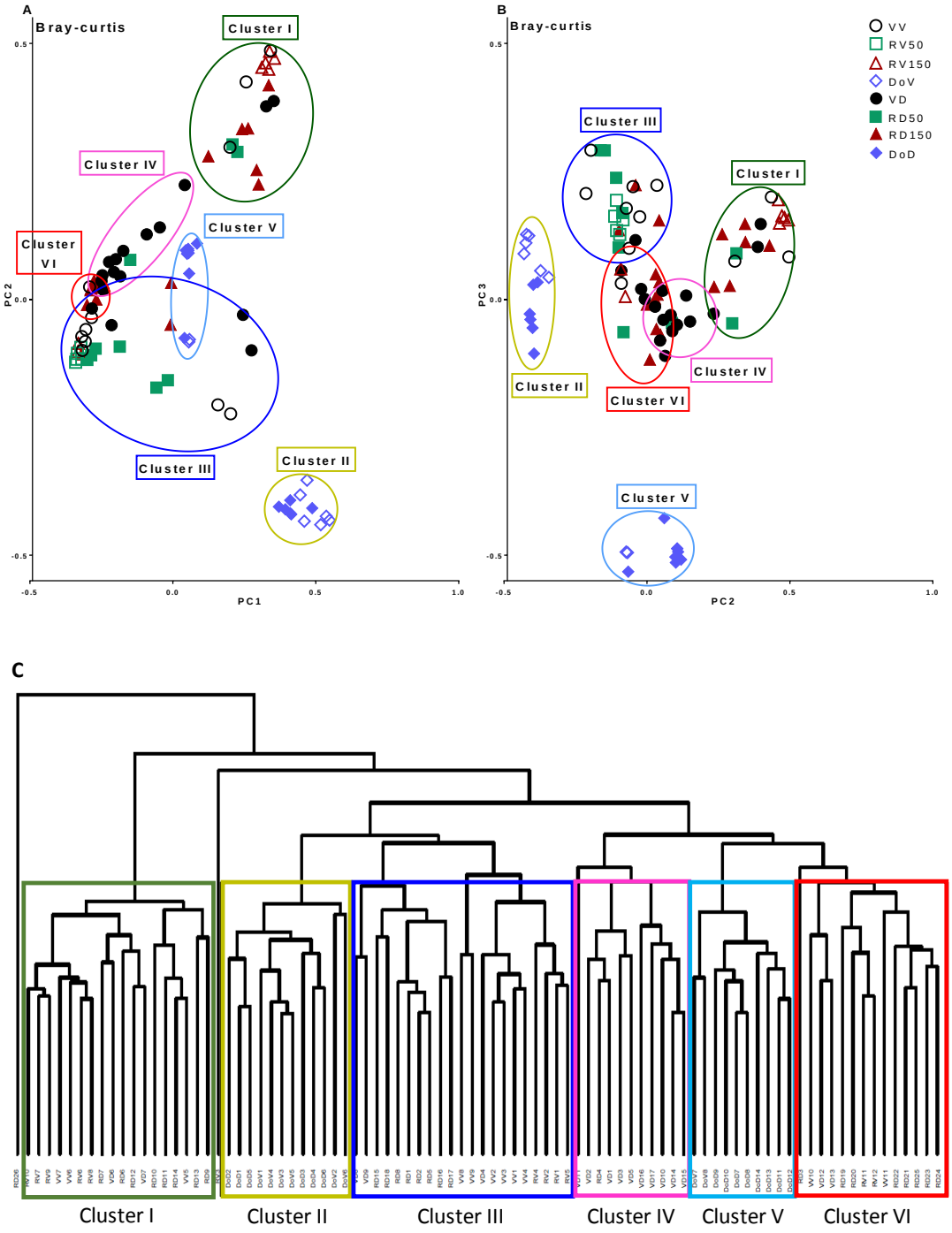


Figure 10. (A, B) Beta diversity (principal coordinate analysis, PCoA). PCoA was calculated with the Bray-curtis distance on two dimensions. Each symbol represents a sample. The axes show the percentage of variation explained by (A): PC1 and PC2; and (B): PC2 and PC3. (C) Hierarchical cluster tree built using the same UniFrac distance matrix used for the PCoA. The effect of the different treatments led to the group of samples within six clusters (I to VI), as described in the text.

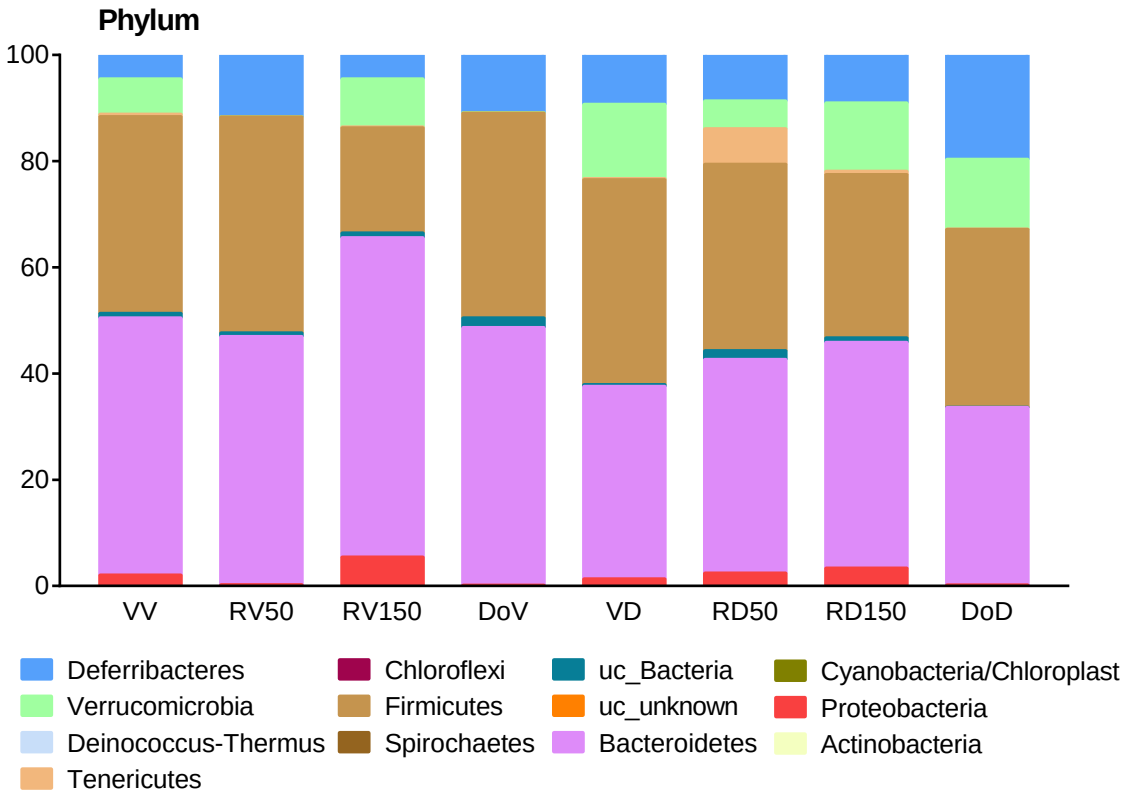


Figure 11. Relative abundance (average) of most common taxa at the phylum level detected in the different experimental groups. Note that unclassified taxa are represented in the 'uc_' category.

At the genus level, ceco-colonic microbiota was dominated by *Parabacteroides* spp. (21.8%), unclassified_Lachnospiraceae (20.9%), *Barnesiella* spp. (14.7%) and *Akkermansia* (6.6%) (Figs. 15 and 16). Less abundant genus (<5%) included unclassified_Clostridiales, *Oscillibacter* spp., *Mucispirillum* spp., *Alistipes* spp., unclassified_Porphyromonadaceae, *Bacteroides* spp. and *Clostridium_XlVa* spp. (Fig. 15). Overall, 155 OTUs were detected among the different experimental groups; but only 87 ± 4 OTUs were present in non-inflamed, vehicle-treated animals (Fig. 17). From those, 31 OTUs had relative abundances higher than 0.5%, and altogether comprised 86% of the microbiota. The more abundant OTUs (relative abundance >5%) included: OTU01 *Parabacteroides* (21.3%), OTU06 uc_Lachnospiraceae (8.7%), OTU03 *Akkermansia* (6.5%), OTU08 *Barnesiella* (4.8%) and OTU02 *Mucispirillum* (4.5%) (Fig. 18).

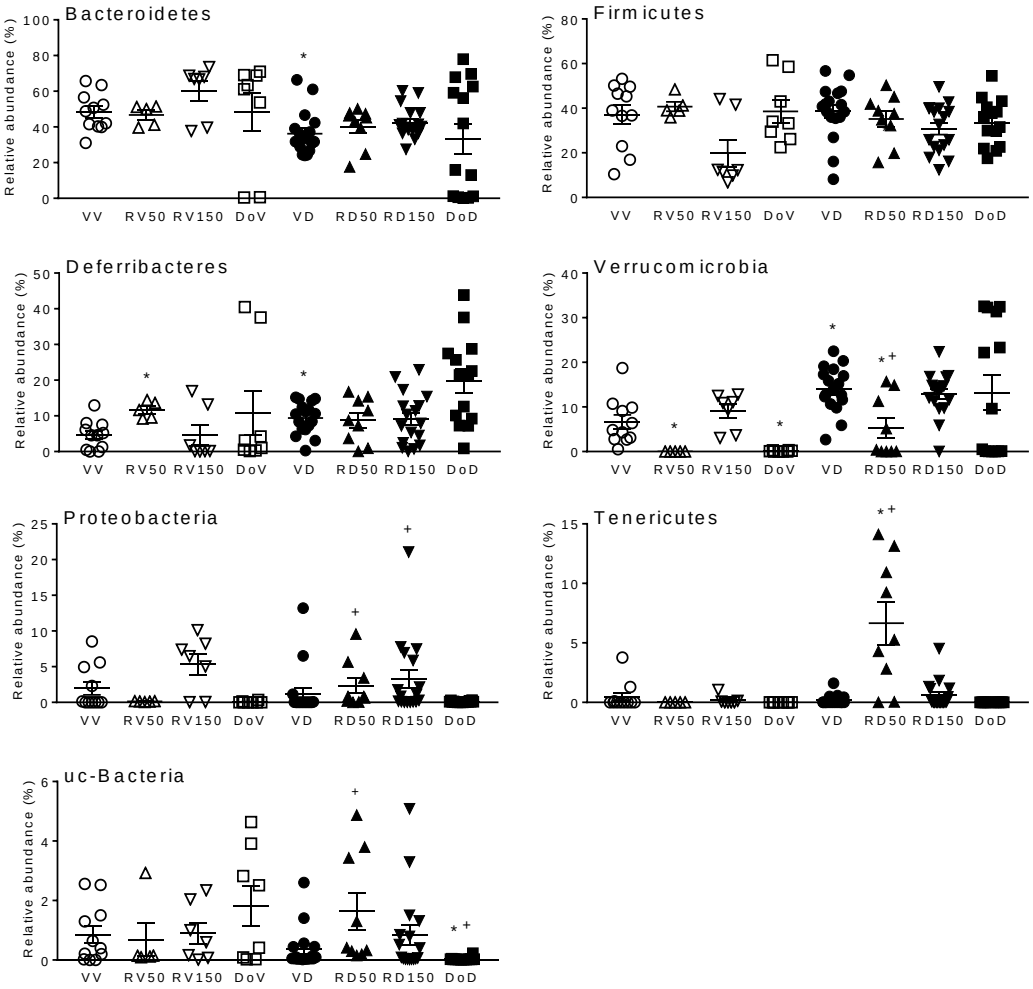
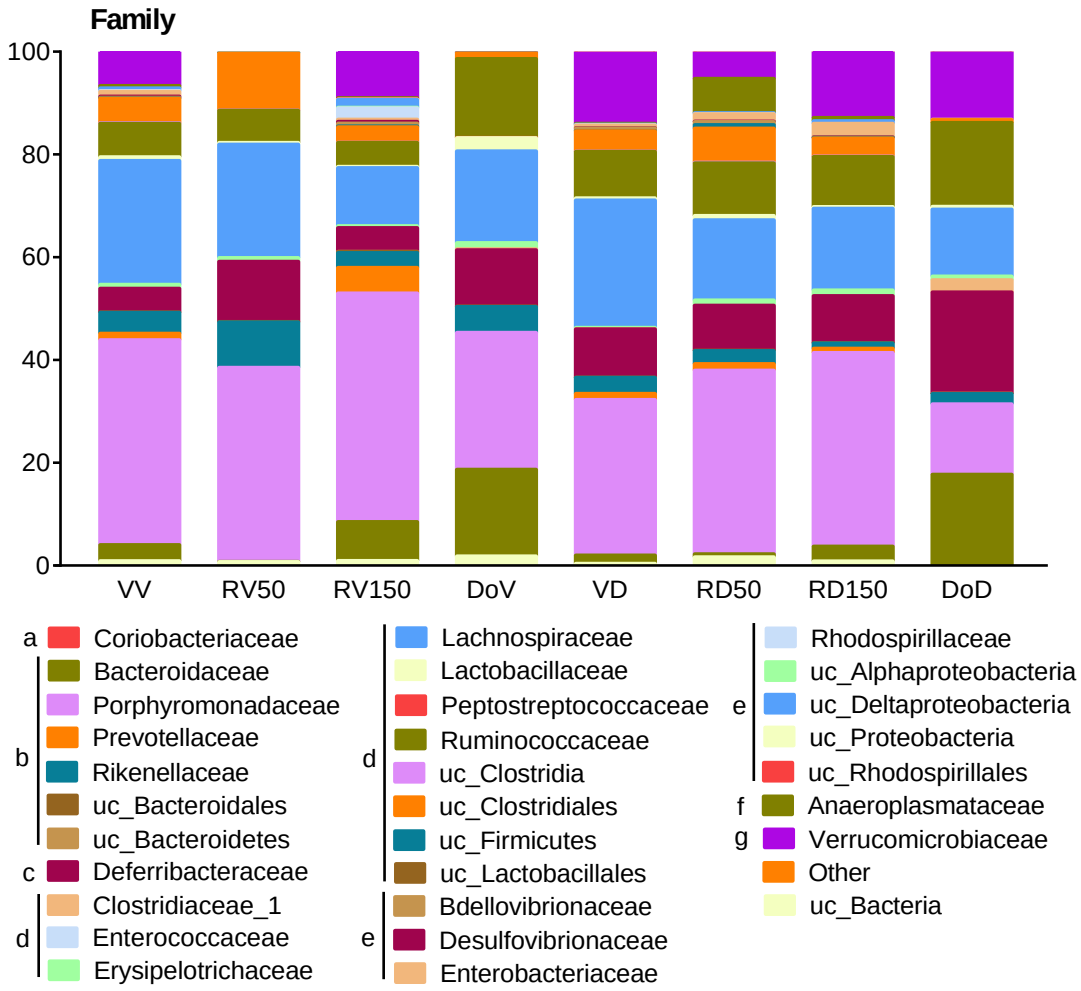


Figure 12. Relative abundance of representative phyla in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$ vs. respective vehicle group; +: $p<0.05$ vs. VD group (Wilcoxon two-sided test). VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.



Phylum: a: Actinobacteria; b: Bacteroidetes; c: Deferribacteres; d: Firmicutes; e: Proteobacteria; f: Tenericutes; g: Verrucomicrobia

Figure 13. Relative abundance (average) of most common taxa at the family level detected in the different experimental groups. Note that taxa unclassified are represented in the 'uc_' category. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

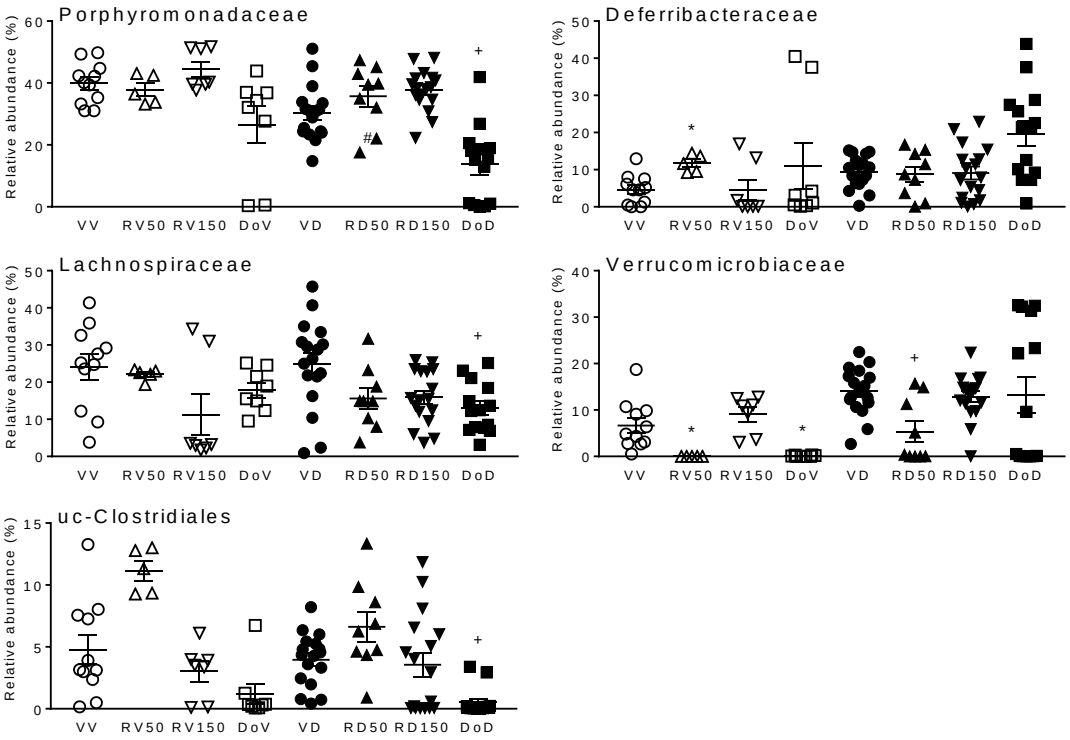
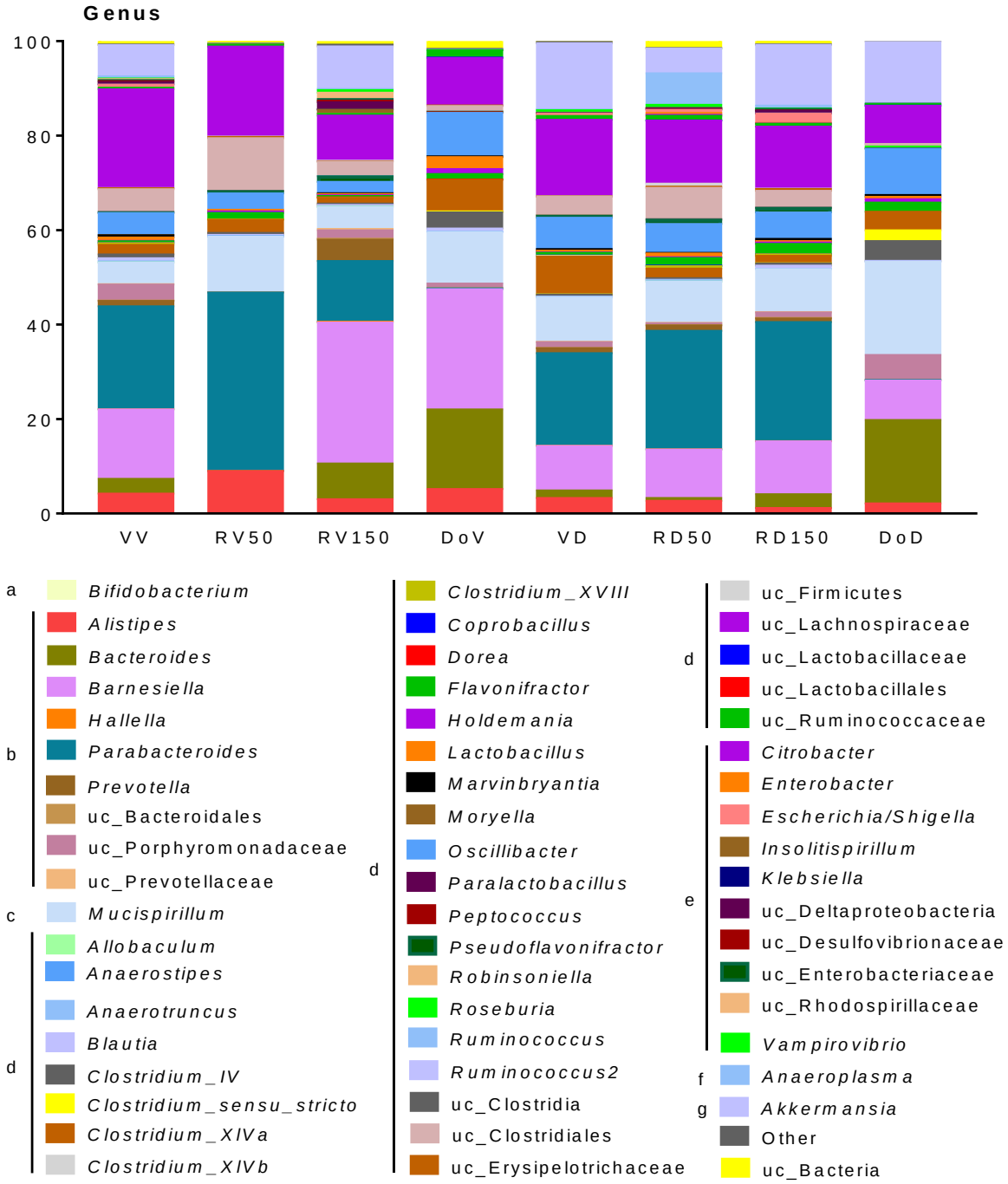


Figure 14. Relative abundance of representative family in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$ vs. respective vehicle group; +: $p<0.05$ vs. VD group (Wilcoxon two-sided test). VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.



Phylum: a: Actinobacteria; b: Bacteroidetes; c: Deferribacteres; d: Firmicutes; e: Proteobacteria; f: Tenericutes; g: Verrucomicrobia

Figure 15. Relative abundance (average) of most common taxa at the genus level detected in the different experimental groups. Note that taxa unclassified are represented in the 'uc_' category. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

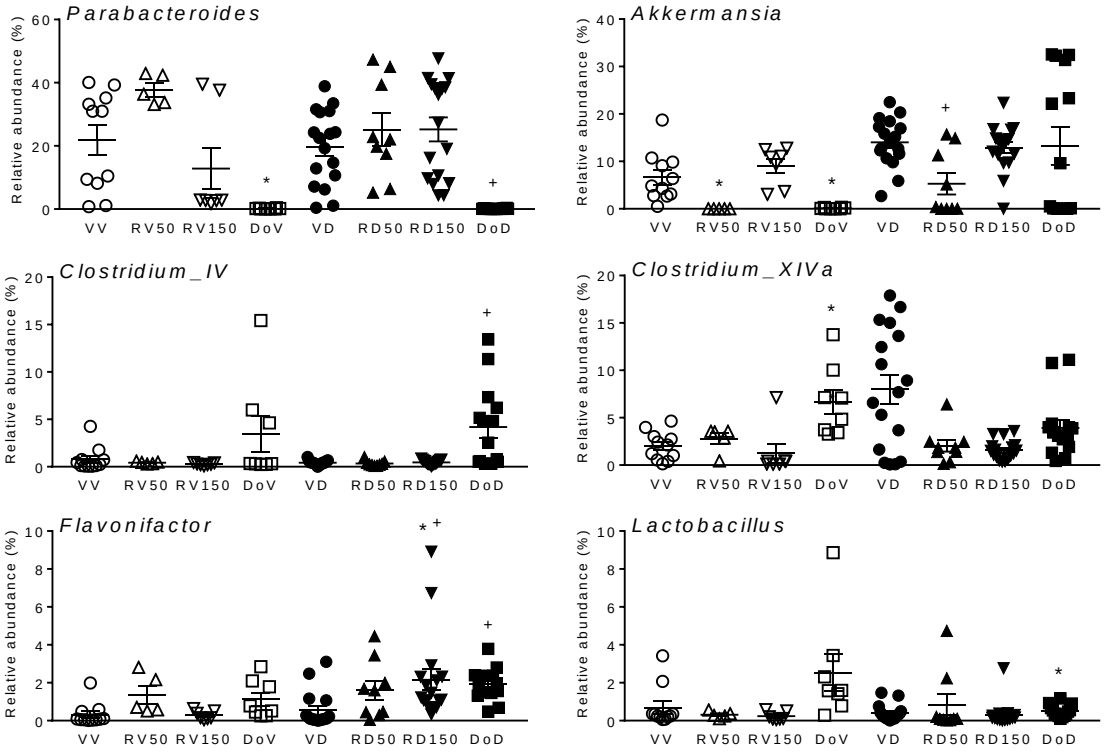


Figure 16. Relative abundance of representative genus in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$ vs. respective vehicle group; +: $p<0.05$ vs. VD group (Wilcoxon two-sided test). VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

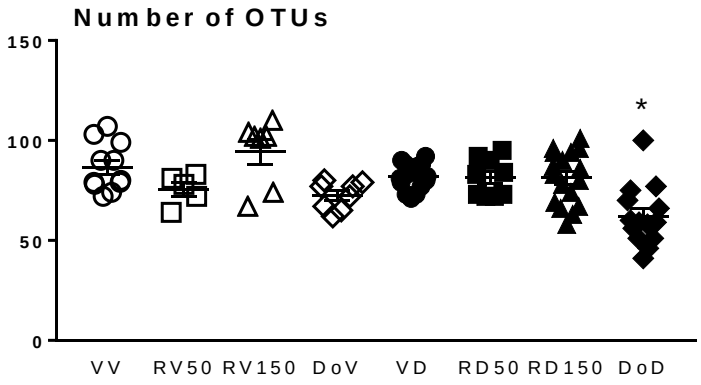


Figure 17. Number of OTUs detected per sample in the different experimental groups. Each symbol represent an individual animal; the horizontal lines with errors represent the mean ± SEM. *: $p<0.05$.VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

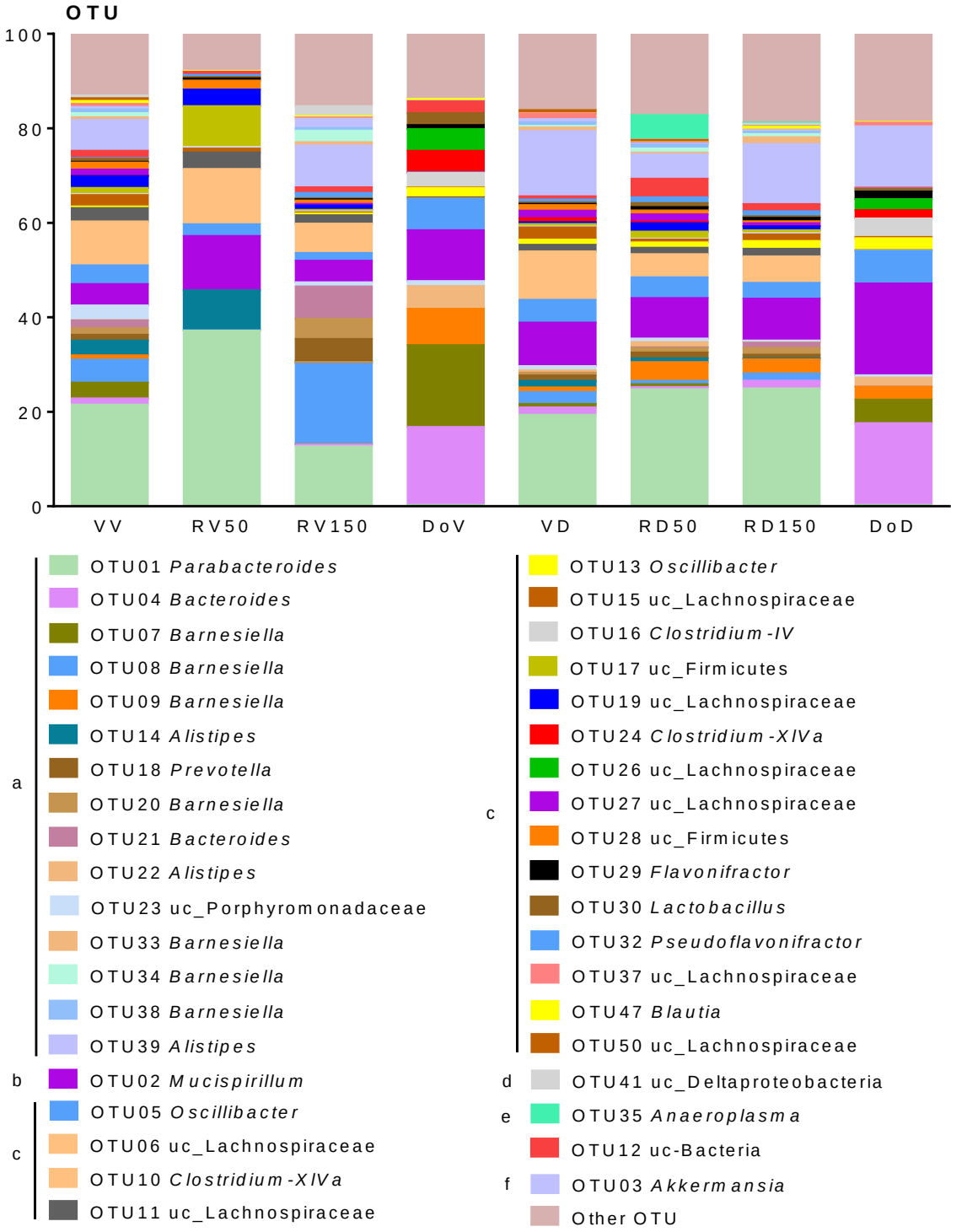


Figure 18. Relative abundance (>0.5%) of OTUs in the different experimental groups. 'uc_': unclassified; VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle; RV150: rifaximin at 150 mg/kg-vehicle; DoV: doxycycline-vehicle; VD: vehicle-DSS; RD50: rifaximin at 50 mg/kg-DSS; RD150: rifaximin at 150 mg/kg-DSS; DoD: doxycycline-DSS.

In vehicle-treated mice, exposure to DSS led to significant microbial changes at the phylum level, characterized by an increase in Verrucomicrobia (14.0%; $p<0.05$ vs. vehicle-vehicle: 6.6%) and Deferribacteres (9.4%; $p<0.05$ vs. vehicle-vehicle: 4.6%) and a simultaneous decrease in Bacteroidetes (36.2%; $p<0.05$ vs. vehicle-vehicle: 48.4%) (Figs. 11 and 12; Table 2). These changes did not manifest as significant variations at the family and genus levels (Figs. 13, 14, 15, 16 and 19A; Table 2). Moreover, a minor decrease in the total number of OTUs detected was also observed (82 ± 1; $p>0.05$ vs. vehicle-vehicle; Fig. 17). In this case, representative OTUs (relative abundance >5%) explained 89% of the microbiota. When compared with vehicle-treated, non-inflamed animals, significant variations (both increases and decreases) were observed in the relative abundance of specific OTUs (Fig. 18, 19B and 19C; Table 2).

Table 2. Effects of DSS-induced colitis on ceco-colonic microbiota in mice.[1]

Phylum	Family	Genus	OTU
Bacteroidetes ↓	Bacteroidaceae =	*Bacteroides* =	OTU21 ↓
Deferribacteres ↑			
Firmicutes =	Lachnospiraceae =	*Clostridium_XlVa* =	OTU10 ↑
		uc_Lachnospiraceae =	OTU19 ↓
			OTU78 ↑
			OTU79 ↑
			OTU147 ↑
	uc_Clostridiales =	uc_Clostridiales =	OTU109 ↑
Verrucomicrobia ↑	Verrucomicrobiaceae =	*Akkermansia* =	OTU03 ↑

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing vehicle-treated animals with and without inflammation.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

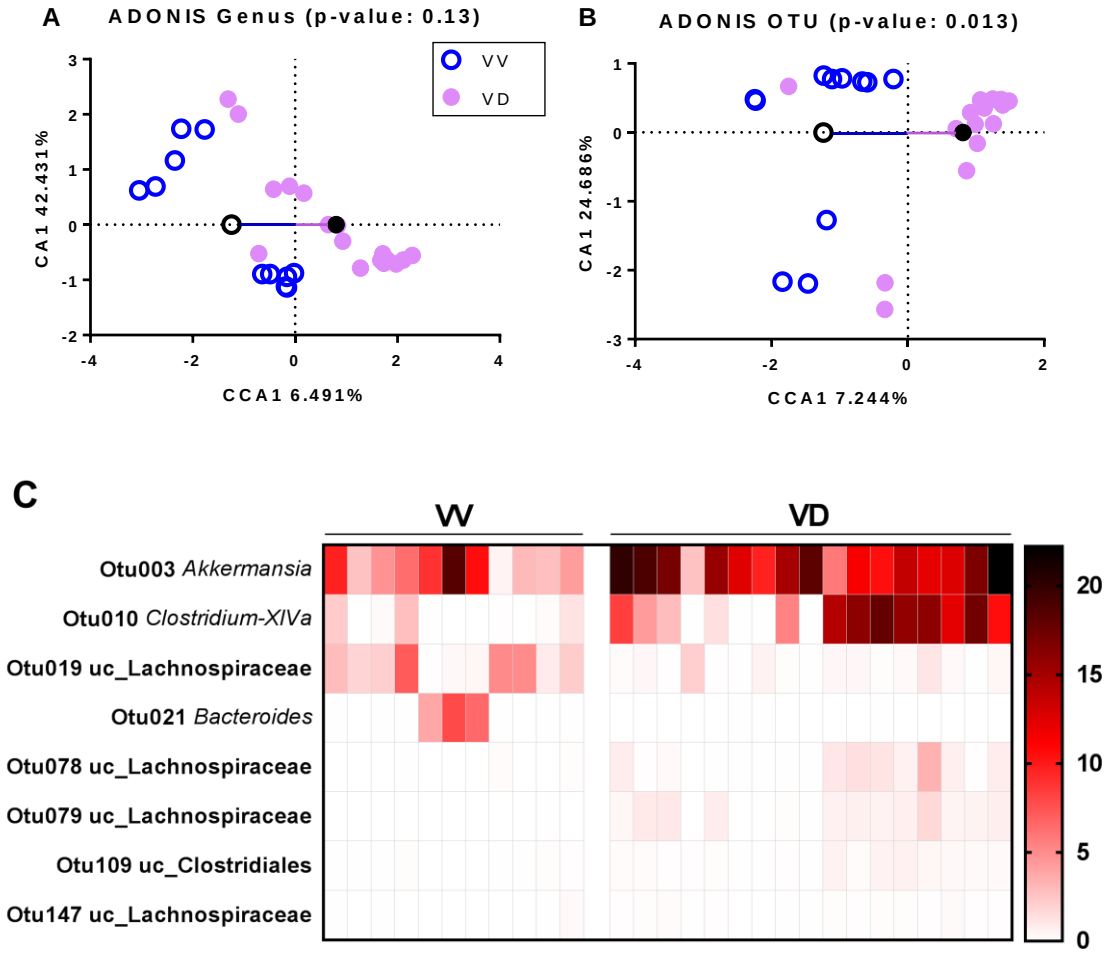


Figure 19. Changes in ceco-colonic microbiota associated to DSS-induced colitis in mice. Permutational multivariate ANOVA (Adonis PERMANOVA) based on sample distances was used to test for changes in the community composition on the genus (A) and OTU levels (B). C: Heatmap representing the relative abundance (%) of OTUs significantly affected during DSS-induced colitis. See also Table 2. VV: vehicle-vehicle; VD: vehicle-DSS.

In non-inflamed animals, rifaximin pre-treatment elicited dose-related changes in the composition of ceco-colonic microbiota, although, as mentioned above, overall biodiversity (alpha and beta diversity) was not affected (Figs. 9 and 10). Most of the changes observed corresponded to the dose of 50 mg/kg, with only minor variations associated to the 150 mg/kg dose. At 50 mg/kg, a decrease in Verrucomicrobia relative abundance (0.02%; $p<0.05$ vs. vehicle-vehicle: 6.6%) and an increase in Deferribacteres relative abundance (11.7%; $p<0.05$ vs. vehicle-vehicle: 4.6%) were detected at the phylum level (Figs. 11, 12; Table 3). At lower taxonomic levels, these changes translated in specific variations affecting the relative abundance

of few bacterial groups at the family, genus and OTU levels (Figs. 13, 14, 15, 16, 18 and 20; Table 3). A non-significant decrease in the total number of OTUs was detected (76 ± 3; p>0.05 vs. vehicle-vehicle; Fig. 17); representative OTUs explained 91% of the microbiota (Fig. 18). On the other hand, rifaximin at the dose of 150 mg/kg did not affect bacterial composition neither at the phylum nor at the genus nor at the OTU levels (Figs. 11, 12, 15, 16, 18 and 21). Only an increase in the relative abundance of the family Rhodospirillaceae (2.2%; P<0.5 vs. vehicle-vehicle: 0.2%) was detected (Figs. 13; Table 3). In this case, 94 ± 6 OTUs were detected (p>0.05 vs. vehicle-vehicle; Fig. 17) and representative OTUs (relative abundance >5%) explained 84% of the microbiota (Fig. 18).

Table 3. Effects of rifaximin on ceco-colonic microbiota in non-inflamed mice.[1]

Phylum	Family	Genus	OTU
Rifaximin (50 mg/kg)			
Actinobacteria =	Bifidobacteriaceae ↑	*Bifidobacterium* ↑	OTU299 ↑
Bacteroidetes =	Rikenellaceae =	*Alistipes* =	OTU23 ↓
		uc_Porphyromonadaceae =	OTU328 ↑
Deferribacteres ↑	Deferribacteres ↑	*Mucispirillum* =	OTU02 ↑
			OTU172 ↑
Firmicutes =	Erysipelotrichaceae =	*Clostridium_XVIII* =	OTU254 ↑
		Holdemania =	OTU43 ↑
	Lachnospiraceae =	*Anaerostipes* =	OTU173 ↑
		Clostridium_XlVa =	OTU24 ↓
			OTU57 ↓
		uc_Lachnospiraceae =	OTU106 ↑
			OTU148 ↑
			OTU27 ↓
	Ruminococcaceae =	*Flavonifractor* =	OTU75 ↑
		Oscillibacter =	OTU13 ↓
	uc_Clostridiales =	uc_Clostridiales =	OTU17 ↑
			OTU63 ↑
	uc_Firmicutes ↓		
Verrucomicrobia ↓	Verrucomicrobiaceae ↓	*Akkermansia* ↓	OTU03 ↓
Rifaximin (150 mg/kg)			
Proteobacteria =	Rhodospirillaceae ↑		

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing rifaximin (50 or 150 mg/Kg)-treated, non-inflamed animals with vehicle-treated animals without inflammation.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

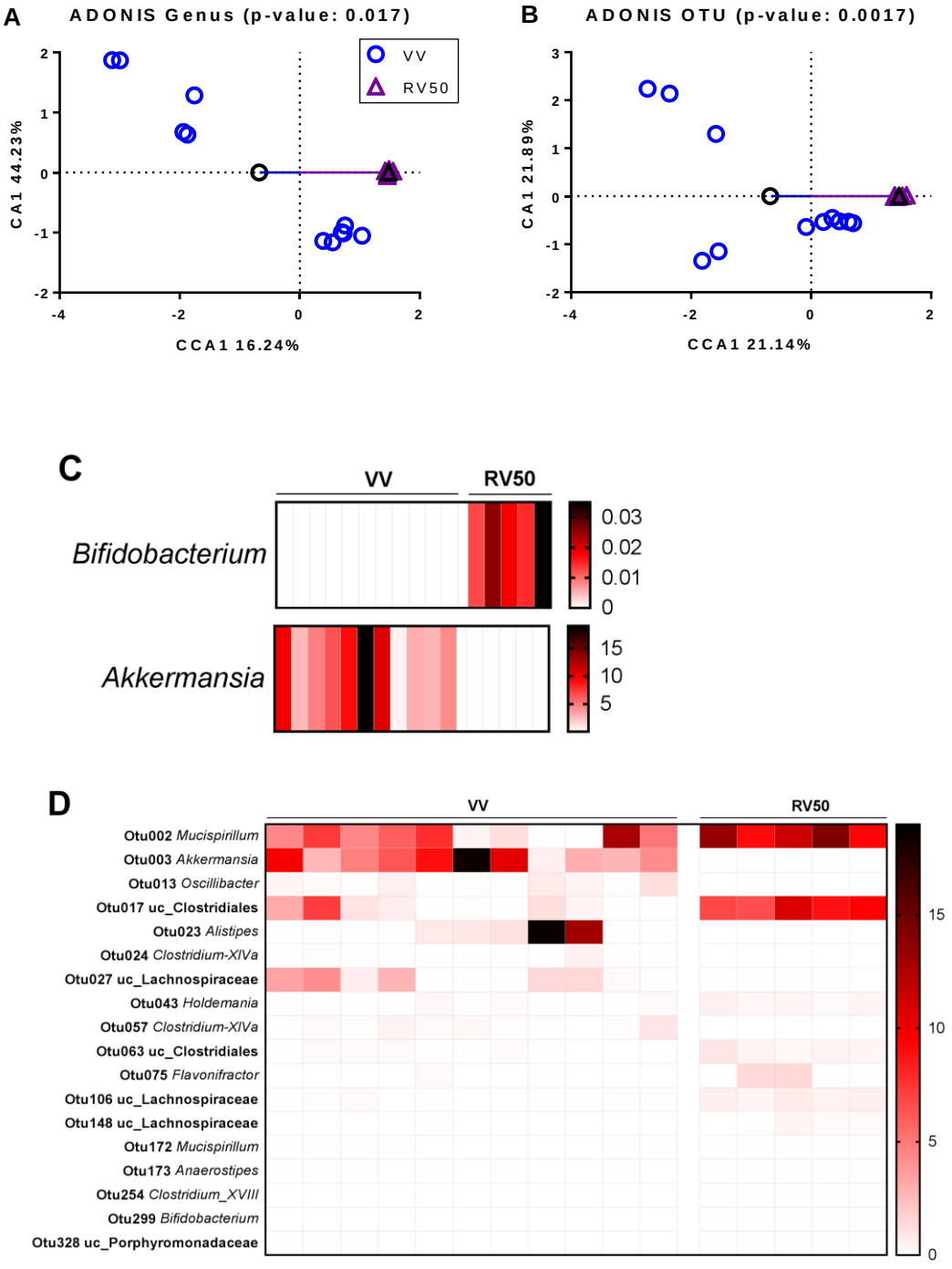


Figure 20. Changes in ceco-colonic microbiota associated to the treatment with rifaximin (50 mg/kg) in non-inflamed mice. Permutational multivariate ANOVA (Adonis PERMANOVA) based on sample distances was used to test for changes in the community composition on the genus (A) and OTU levels (B). C: Heatmap representing the relative abundance (%) of genus significantly affected by rifaximin. D: Heatmap representing the relative abundance (%) of OTUs significantly affected by rifaximin. See also Table 3. VV: vehicle-vehicle; RV50: rifaximin at 50 mg/kg-vehicle.

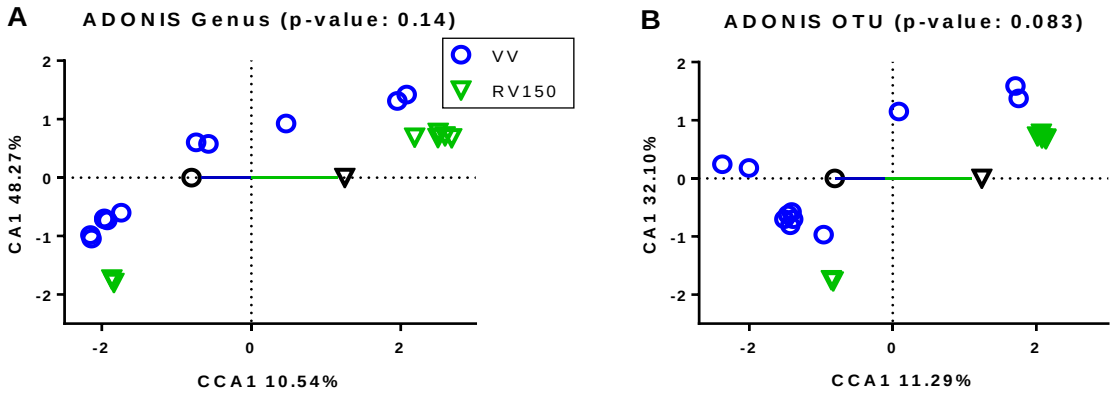


Figure 21. Changes in ceco-colonic microbiota associated to the treatment with rifaximin (150 mg/kg) in non-inflamed mice. Permutational multivariate ANOVA (Adonis PERMANOVA) based on sample distances was used to test for changes in the community composition on the genus (A) and OTU levels (B). See also Table 3. VV: vehicle-vehicle; RV150: rifaximin at 150 mg/kg-vehicle.

In animals pre-treated with rifaximin, regardless the dose, and exposed to DSS, minor changes in the composition of ceco-colonic microbiota were detected, maintaining a beta diversity similar to that observed in non-inflamed animals pre-treated with the antibiotic (Fig. 10). The total number of OTUs detected (82±3, and 81±3 at 50 and 150 mg/kg, respectively) was similar to that in the respective non-inflamed groups (Fig. 17). Representative OTUs (relative abundance >5%) explained 86% and 88% of the microbiota for the 50 and 150 mg/kg dose, respectively (Fig. 18). Nevertheless, few changes (both increases and decreases) in relative bacterial abundance, at the phylum, family, genus and OTU levels were detected (Figs. 11, 12, 13, 14, 15, 16 and 18; Tables 5 and 7). When compared to inflamed animals pre-treated with vehicle, both beta diversity and relative bacterial abundances were modified in inflamed, rifaximin pre-treated animals. Phylogenetic characterization revealed numerous significant changes in relative bacterial abundances at the phylum, family, genus and OTU levels (Figs. 11, 12, 13, 14, 15, 16 and 18; Tables 4 and 6).

Table 4. Effects of rifaximin (50 mg/kg) on ceco-colonic microbiota during DSS-induced colitis in mice.[1]

Phylum	Family	Genus	OTU
Firmicutes =	Lachnospiraceae =	*Robinsoniella* ↓	
		uc_Lachnospiraceae =	OTU51 ↑
			OTU06 ↑
			OTU74 ↑
	uc_Clostridiales =	uc_Clostridiales =	OTU144 ↑
	Erysipelotrichaceae =	*Clostridium_XVIII* =	OTU254 ↑
	uc_Firmicutes ↑	uc_Firmicutes ↑	
Proteobacteria ↑	Enterobacteriaceae =	uc_Enterobacteriaceae ↑	OTU53 ↑
Tenericutes ↑	Anaeroplasmataceae ↑	*Anaeroplasma* ↑	OTU35 ↑
Verrucomicrobia ↓	Verrucomicrobiaceae ↓	*Akkermansia* ↓	
uc_Bacteria ↑	uc_Bacteria =	uc_Bacteria =	OTU12 ↑

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing rifaximin (50 mg/kg)-treated, inflamed animals with vehicle-treated animals with inflammation.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

Table 5. Changes in ceco-colonic microbiota associated to colitis in rifaximin (50 mg/kg)-treated mice.[1]

Phylum	Family	Genus	OTU
Actinobacteria ↓	Bifidobacteriaceae ↓	*Bifidobacterium* ↓	OTU299↓
Bacteroidetes =	Porphyromonadaceae =	*Barnesiella* =	OTU09 ↑
	Rikenellaceae =	*Alistipes* =	OTU360↓
Firmicutes =	Lachnospiraceae =	*Clostridium_XlVa* =	OTU58 ↓
		uc_Lachnospiraceae =	OTU65 ↓
			OTU106↓
			OTU203↓
	Ruminococcaceae =	*Oscillibacter* =	OTU13 ↑
			OTU49 ↑
Tenericutes ↑	Anaeroplasmataceae ↑	*Anaeroplasma* =	OTU35 ↑
Verrucomicrobia ↑			

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing rifaximin (50 mg/kg)-treated, inflamed animals with rifaximin (50 mg/Kg)-treated, non-inflamed animals.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

Table 6. Effects of rifaximin (150 mg/kg) on ceco-colonic microbiota during DSS-induced colitis mice.[1]

Phylum	Family	Genus	OTU
Bacteroidetes =	Porphyromonadaceae ↓	*Barnesiella* =	OTU69 ↑
		Parabacteroides =	
Firmicutes =	Ruminococcaceae =	*Anaerotruncus* ↓	
		Flavonifractor ↑	OTU29 ↑
			OTU40 ↑
		Pseudoflavonifractor ↑	
	Enterococcaceae ↑	*Enterococcus* ↑	
	Peptostreptococcaceae ↓	*Clostridium_XI* ↓	
	Lachnospiraceae =	*Blautia* ↑	OTU47 ↑
			OTU133 ↑
		Clostridium_XlVb ↓	OTU131 ↓
		Clostridium_XlVa =	OTU10 ↓
			OTU36 ↑
		Robinsoniella ↓	
		uc_Lachnospiraceae =	OTU078 ↓
			OTU079 ↓
			OTU27 ↓
			OTU51 ↑
			OTU70 ↓
	Erysipelotrichaceae ↑	*Clostridium_XVIII* =	OTU175 ↑
		Holdemania ↑	OTU43 ↓
		Turicibacter ↓	
		Coprobacillus ↑	
		uc_Erysipelotrichaceae ↑	OTU67 ↑
	uc_Clostridiales =	uc_Clostridiales =	OTU100 ↓
Proteobacteria ↑	Enterobacteriaceae ↑	*Citrobacter* ↑	
		uc_Deltaproteobacteria =	OTU41 ↑
		Enterobacter ↑	
		Klebsiella ↑	
		uc_Enterobacteriaceae ↑	OTU53 ↑
	uc_Desulfovibrionales =	uc_Desulfovibrionales ↑	

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing rifaximin (150 mg/kg)-treated, inflamed animals with vehicle-treated animals with inflammation. =: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

Table 7. Changes in ceco-colonic microbiota associated to colitis in rifaximin (150 mg/kg)-treated mice.[1]

Phylum	Family	Genus	OTU
Bacteroidetes =	Porphyromonadaceae =	*Barnesiella* =	OTU142 ↓
Firmicutes =	Clostridiales_uc =	uc_Clostridiales =	OTU270 ↓
	Ruminococcaceae =	*Ruminococcus* ↓	OTU181 ↓
		Flavonifractor ↑	
Proteobacteria =	Enterobacteriaceae =	*Enterobacter* ↑	
	Rhodospirillaceae ↓	*Insolitispirillum* ↓	
		uc_Rhodospirillales ↓	
	uc_Alphaproteobacteria ↓	uc_Alphaproteobacteria ↓	
	uc_Rhodospirillaceae ↓	uc_Rhodospirillaceae ↓	OTU055 ↓

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing rifaximin (150 mg/kg)-treated, inflamed animals with rifaximin (150 mg/Kg)-treated, non-inflamed animals.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

Doxycycline alters the ceco-colonic microbiota: Phylogenetic analysis

Doxycycline treatment in non-inflamed animals was associated to profound alterations in microbial composition, affecting all phylogenetical levels (Table 8). Overall, the more prominent change was a reduction of the phylum Verrucomicrobia (0.1%; $P<0.5$ vs. vehicle-vehicle: 6.6%) (Figs. 11 and 12). At the genus level, there were extensive changes in the relative abundance of several genus included within the Firmicutes phylum, although at the phylum level per se no changes were detected (Figs. 15, 16, 22A and 22C; Table 8). At the OTU level, these changes translated in a small, but significant, decrease in the number of OTUs detected (72 ± 3; $p<0.05$ vs. vehicle-vehicle group; Fig. 17), with representative OTUs (relative abundance >5%) explaining 92% of the microbiota. Extensive variations (increase or decrease) in the relative abundance of specific OTUs were observed (Figs. 18, 22B and 22D; Table 8).

Table 8. Effects of doxycycline on ceco-colonic microbiota in non-inflamed mice.[1]

Phylum	Family	Genus	OTU
Bacteroidetes =	Bacteroidaceae =	*Bacteroides* =	OTU04 ↑
			OTU208 ↑
	Porphyromonadaceae =	*Parabacteroides* ↓	OTU01 ↓
		Barnesiella =	OTU07 ↑
			OTU09 ↑
	Rikenellaceae =	*Alistipes* =	OTU22 ↑
Firmicutes =	Lachnospiraceae =	*Blautia* =	OTU133 ↑
		Clostridium_XlVa ↑	OTU58 ↑
			OTU114 ↑
		Moryella ↓	
		Roseburia ↑	OTU105 ↑
		Ruminococcus2 ↑	
		uc_Lachnospiraceae =	OTU27 ↓
			OTU26 ↑
			OTU48 ↑
			OTU50 ↓
			OTU54 ↑
			OTU65 ↑
			OTU108 ↓
			OTU129 ↓
			OTU160 ↑
			OTU197 ↓
			OTU207 ↓
			OTU209 ↑
		Allobaculum ↓	
		Holdemania ↑	OTU43 ↑
		Lactobacillus =	OTU30 ↑
	Peptococcaceae_1 =	*Peptococcus* ↑	OTU159 ↑
	Ruminococcaceae =	*Anaerotruncus* ↓	OTU87 ↓
		Flavonifractor =	OTU29 ↑
		uc_Ruminococcaceae =	OTU116 ↓
			OTU125 ↑
	uc_Clostridiales =	uc_Clostridiales =	OTU17 ↓
			OTU28 ↓
			OTU100 ↓
uc_Bacteria =	uc_Bacteria =	uc_Bacteria =	OTU97 ↑
			OTU263 ↑
Verrucomicrobia ↓	Verrucomicrobiaceae ↓	*Akkermansia* ↓	

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing doxycycline-treated, non-inflamed animals with vehicle-treated animals without inflammation.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

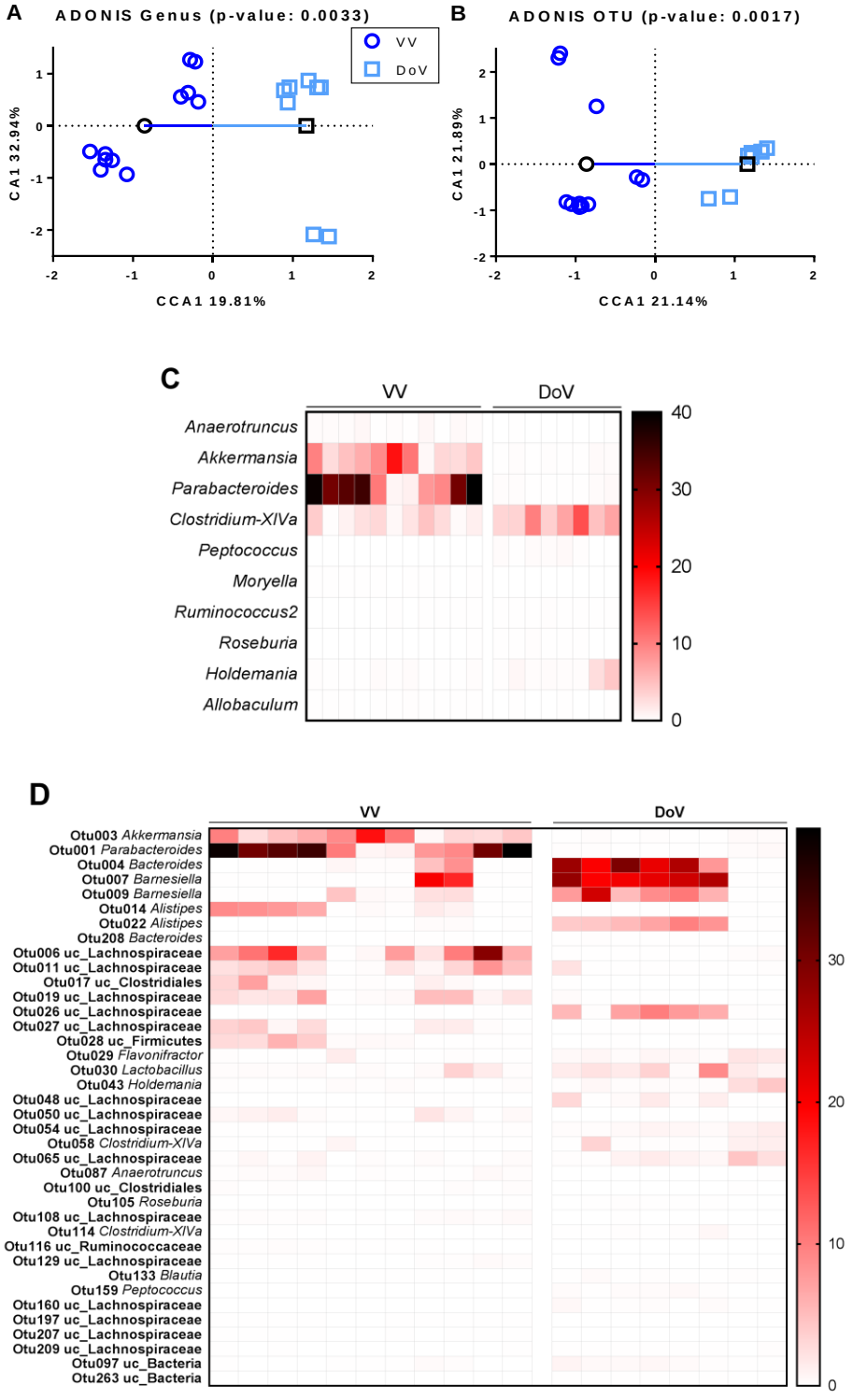


Figure 22. Changes in ceco-colonic microbiota associated to the treatment with doxycycline in non-inflamed mice. Permutational multivariate ANOVA (Adonis PERMANOVA) based on sample distances was used to test for changes in the community composition on the genus (A) and OTU levels (B).C: Heatmap representing the relative abundance (%) of genus significantly affected by doxycycline. D: Heatmap representing the relative abundance (%) of OTUs significantly affected by doxycycline. See also Table 7. VV: vehicle-vehicle; DoV: doxycycline-vehicle.

Doxycycline-pre-treated animals exposed to DSS showed similar ceco-colonic microbial composition to that described above for non-inflamed animals pre-treated with the antibiotic. Nevertheless minor, but significant, variations at the family, genus and OTU levels were detected (Figs. 15, 16, 18 and 19; Table 9). Moreover, an increase in the relative abundance of the phylum Verrucomicrobia was observed in inflamed animals; however, due to the relative large variability observed, no statistical significance was reached (Figs. 11 and 12; Table 9).

Compared with vehicle-treated animals with colitis, profound changes were detected in the ceco-colonic microbiota of doxycycline-treated animals with inflammation. Significant changes were detected at the family, genus and OTU levels (Figs. 13, 14, 15, 16 and 18; Table 10). In inflamed animals pre-treated with doxycycline, the number of OTUs detected was 62±4; ($p>0.05$ vs. vehicle-DSS group) and representative OTUs (relative abundance >5%) explained 87% of the microbiota (Fig. 18).

Table 9. Changes in ceco-colonic microbiota associated to colitis in doxycycline-treated mice.[1]

Phylum	Family	Genus	OTU
Firmicutes =	Lachnospiraceae =	*Anaerostipes* ↓	OTU173 ↓
		uc_Lachnospiraceae ↓	OTU65 ↓
			OTU203 ↓
	Lactobacillaceae ↓	*Lactobacillus* ↓	
		Paralactobacillus ↓	
		uc_Lactobacillaceae ↓	
	Peptococcaceae_1 ↓	*Peptococcus* ↓	OTU159 ↓
	Ruminococcaceae =	*Anaerotruncus* ↑	
	uc_Clostridia ↓	uc_Clostridia ↓	
	uc_Firmicutes ↓	uc_Firmicutes ↓	
	uc_Lactobacillales ↓	uc_Lactobacillales ↓	
uc_Bacteria ↓	uc_Bacteria ↓	uc_Bacteria ↓	

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing doxycycline-treated, inflamed animals with doxycycline-treated, non-inflamed animals.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

Table 10. Effects of doxycycline on ceco-colonic microbiota during DSS-induced colitis in mice.[1]

Phylum	Family	Genus	OTU
Bacteroidetes =	Porphyromonadaceae ↓	*Parabacteroides* ↓	OTU01 ↓
			OTU247 ↓
	Prevotellaceae =	*Hallella* ↓	
	Rikenellaceae =	*Alistipes* =	OTU14 ↓
Firmicutes =	Ruminococcaceae =	*Anaerotruncus* ↓	OTU87 ↓
		Flavonifractor ↑	OTU29 ↑
			OTU143 ↓
		Butyricicoccus ↓	
		Clostridium_IV ↑	OTU16 ↑
		uc_Ruminococcaceae =	OTU119 ↓
			OTU116 ↓
			OTU125 ↑
	Lachnospiraceae ↓	*Blautia* ↑	
		Clostridium_XlVb ↓	OTU131 ↓
		Robinsoniella ↓	
		Moryella ↓	
		Oribacterium ↓	
		Parasporobacterium ↓	
		Roseburia ↑	OTU105 ↑
		uc_Lachnospiraceae ↓	OTU27 ↓
			OTU37 ↓
			OTU50 ↓
			OTU52 ↓
			OTU54 ↑
			OTU70 ↓
			OTU72 ↓
			OTU95 ↓
			OTU108 ↓
			OTU129 ↓
			OTU147 ↓
			OTU151 ↓
		Clostridium_XlVa =	OTU10 ↓
			OTU58 ↑
			OTU114 ↑
	uc_Clostridiales ↓	uc_Clostridiales ↓	OTU109 ↓
			OTU28 ↓
			OTU63 ↓
			OTU78 ↓
			OTU79 ↓
			OTU84 ↓
			OTU100 ↓
			OTU17 ↓
	uc_Clostridia ↓	uc_Clostridia ↓	
	Clostridiaceae_1 =	*Clostridium_sensu_stricto* ↑	OTU44 ↑
			OTU113 ↑
		uc_Clostridiaceae_1 ↑	
	Erysipelotrichaceae =	*Clostridium_XVIII* ↓	OTU68 ↓
		Holdemania ↑	OTU43 ↑
		Turicibacter ↓	
		Coprobacillus ↓	OTU121 ↓
	uc_Firmicutes ↓	uc_Firmicutes ↓	
Proteobacteria =	Enterobacteriaceae =	*Klebsiella* ↑	
		uc_Enterobacteriaceae =	OTU53 ↑
Tenericutes =	Anaeroplasmataceae =	*Anaerobacter* ↑	
		Anaeroplasma ↓	
uc_Bacteria ↓	uc_Bacteria ↓	uc_Bacteria ↓	OTU219 ↓

[1]: Data show taxonomic levels and OTUs significantly affected (relative abundance) when comparing doxycycline-treated, inflamed animals with vehicle-treated animals with inflammation.
=: No significant change in relative abundance. ↑: Significant increase in relative abundance. ↓: Significant decrease in relative abundance.

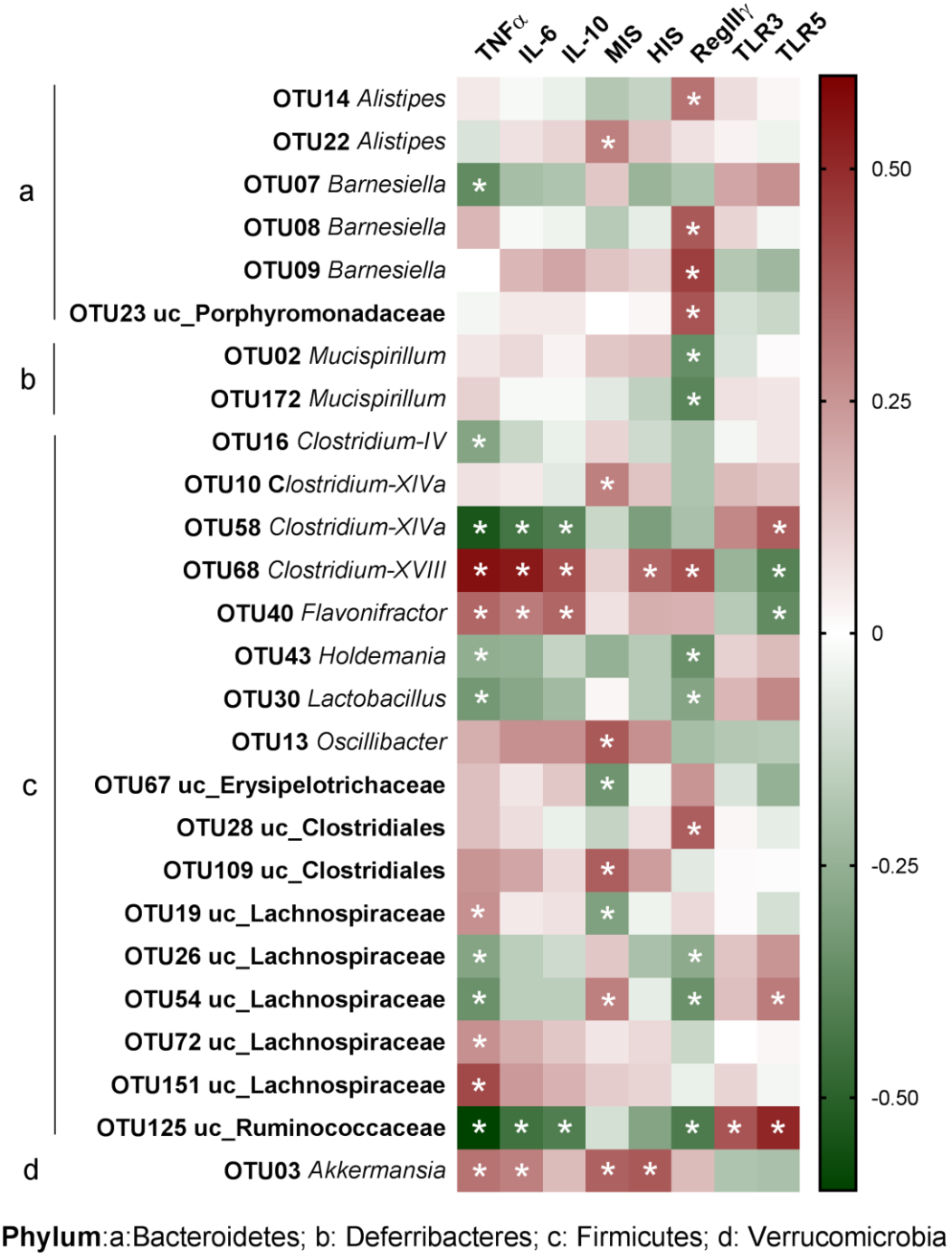


Figure 23. Correlations between microbiota abundances and inflammatory scores and gene expression of immune-related markers. Only significantly modified OTUs during rifaximin treatment were analyzed. Spearman correlation coefficient was used. *: $p<0.05$, indicates a statistically significant correlation, negative or positive according to the color scale. TNFα, Tumor necrosis factor alpha; IL-6, interleukin-6; IL-10, interleukin-10; MIS, macroscopic inflammatory score; HIS, histopathological inflammatory score; RegIIIɣ, regenerating islet-derived protein 3 gamma; TLR, Toll-like receptor; OTU: operational taxonomic unit.

Intercorrelation between gut microbiota and inflammatory scores and immune-related markers

Fig. 23 summarizes the correlations detected among microbiota, at the OTU level, and inflammatory scores and immune-related markers. Only OTUs that were significantly modified by antibiotic treatment are represented.

Only inflammatory scores (macroscopic or microscopic) and gene expression of TNFα, IL-6, IL-10, RegIIIγ and TLR3 and 5 showed some correlations with specific bacterial groups. Overall, correlations, either positive or negative, were mainly detected with the phyla Bacteroidetes and Firmicutes.

Discussion

In this study, we evaluated, in a model of colitis, the antimicrobial activity and the potential immunomodulatory and anti-inflammatory effects associated to rifaximin. For this, we assessed the clinical, histopathological, molecular and microbiological effects of the non-absorbable, wide spectrum antibiotic rifaximin on intestinal inflammation in the DSS-induced colitis model in mice. Overall, the results obtained show that rifaximin did not affect the clinical course of colitis nor the histopathological alterations and immune-related changes associated to the process. Moreover, as it relates to the microbiota, rifaximin showed also limited modulatory effects, either in normal condition or during inflammation, as assessed by 16S rRNA gene sequencing.

In mice, DSS exposure elicited a state of inflammation, with clinical alterations and a local immune activation, consistent with previous reports using the same model.[27,34–36] Preventive treatment with rifaximin, irrespective of the dose considered, failed to attenuate colitis-associated clinical signs and the indices of colonic inflammation, as assessed at necropsy. Moreover, a tendency to increase body weight loss was observed in rifaximin-treated animals, thus indicating a worsening in their clinical state. Furthermore, histopathological changes associated to colitis were not affected by rifaximin. These observations are in agreement with previous data showing no beneficial effects associated to the treatment with rifaximin during DSS- and DNBS-induced colitis in mice.[23,24] However, they differ from data showing that

rifaximin reduced inflammation severity, body weight loss and histopathological alterations in a model of TNBS-induced colitis in mice.[37] Differences in the experimental conditions, including the colitis induction methods and the sex and/or strain of the animals used might explain these differences. As expected, doxycycline, an antibiotic with proven immunomodulatory and anti-inflammatory activity at pre-clinical and clinical level[22,23,38,39] attenuated some of the clinical signs associated to DSS-induced colitis, although histopathological alterations were not affected. These results partially agree with previous data showing an improvement in the clinical condition and histopathological alterations in mice with DSS-induced colitis.[23]

Beneficial effects of rifaximin in IBD in humans have been related, at least in part, to its ability to modulate the immune response.[40–42] DSS-induced colitis was associated to an immune activation, as indicated by the local up-regulation of pro-inflammatory cytokines, consistent with previous reports.[27,34–36] Treatment with rifaximin either did not affect this state or elicited a further up-regulation, suggesting an enhanced, pro-inflammatory, immune response. These observations are in agreement with published data in the same model, showing that rifaximin was unable to reduce, and in some cases increased, colonic expression of pro-inflammatory cytokines.[14,23] However, positive modulatory effects (reduction in expression levels) have been described for rifaximin during DNBS- and TNBS-induced colitis in mice,[24,37] thus emphasizing the importance of the model used when assessing immune modulation. In the same experimental conditions, we were able to demonstrate the immunomodulatory properties of doxycycline. Indeed, pre-treatment with doxycycline, in line with the aforementioned attenuation of the clinical state, completely normalized immune-related markers in animals receiving DSS. The positive immune modulation observed is similar to that previously described for DNBS- and DSS-induced colitis in mice.[23,24]

As part of the potential immunomodulatory effects of rifaximin, we also assessed changes in the expression of the anti-inflammatory cytokine IL-10. In colitic animals treated with rifaximin, a significantly up-regulation of IL-10 expression was detected, in agreement with previous data.[14] This might be regarded as a compensatory response associated to the enhanced up-regulation observed in the same animals for pro-inflammatory cytokines, as described above. In these conditions, the up-regulation of IL-10 could act as a contention mechanism avoiding an exacerbated inflammatory response and excessive tissue damage.[3,43,44] This might explain why, even though the enhanced pro-inflammatory state, clinical sings, indices of colonic inflammation and histopathological changes were not worsened in rifaximin-treated animals.

Recent evidences suggest that rifaximin might exert its local immunomodulatory effects within the gastrointestinal tract through the activation of a PXR-NF-κB-dependent pathway.[45] The fact that PXR shows significant differences between rodents and humans and that rifaximin acts as an intestinal-specific human PXR agonist[46,47] might explain differences between humans and mice in the immunomodulatory activity of rifaximin and why rifaximin does not exhibit full anti-inflammatory effect in mice models of intestinal inflammation. Indeed, significant immunomodulatory and anti-inflammatory effects of rifaximin were revealed in transgenic PXR-humanized vs. wild-type mice with colitis[14,48] or by activation of murine PXR receptors.[49–52] In this study, we assessed the colonic expression of PXR during DSS-induced colitis, observing a tendency towards a down-regulation similar to that described in other studies using the same model.[49] The mechanism of action of rifaximin seems to include also an up-regulation in the intestinal expression of PXRs,[14,53] however no expression changes were observed during the treatment with rifaximin. Interestingly, PXR expression levels were normalized by doxycycline, further supporting its immunomodulatory and anti-inflammatory activity. Overall, these observations indicate that, due to differences between, murine and human PXRs, mice present limitations in their validity and translatability as animal model to study the mechanisms of action of rifaximin within the gut.

Independently of its antimicrobial activity, part of the immunomodulatory effects of rifaximin might be related to the modulation of host-bacterial interaction systems. To address this possibility, we assessed potential changes in the expression of AMPs as well as the main TLRs expressed in the gut and implicated in the initiation, course and resolution of intestinal inflammation.[54–57] Rifaximin, *per se*, induced minor changes in TLRs expression, with a marginal up-regulation of TLR3 and TLR4 for the lowest dose tested, consistent with our previous observations. This might be related to the limited effects observed for rifaximin on GCM, in agreement with previous data and our previous observations.[19,20,58,59] Exposure to DSS had minor effects on TLRs expression, with only a down-regulation of TLR5. In inflamed animals, treatment with rifaximin resulted in a down regulation of TLR3, 4 and 5, with an up-regulation of TLR7; without consistent dose-relation. Doxycycline, *per se*, did not affect TLRs expression either in healthy or inflamed animals; despite the changes observed in the microbiota (see below). Overall, TLRs expression correlated negatively with other markers of inflammatory activity. It is difficult to establish the relative importance of these observations since changes in TLRs expression might relate to the immune state and/or the characteristics of the microbiota, without clear causal relationships.[60] As it relates to AMPs, the only significant changes were

observed for the expression of RegIIIγ, which was up-regulated during colitis both in non-treated or rifaximin-treated mice. RegIIIγ is considered a mucosal protective factor against specific microbial pathogens.[61] Since intestinal bacteria can activate RegIIIγ expression,[62,63] it is tempting to speculate that selective changes of the normal microbiota, generated during inflammation and/or the treatment with rifaximin might be responsible for RegIIIγ up-regulation, with a general defensive objective. As for other immune-related markers, treatment with doxycycline normalized RegIIIγ expression in inflamed animals. This response is consistent with positive modulatory effects exerted by doxycycline on other epithelial barrier protective factors during states of inflammation,[23,24] further supporting the immunomodulatory and anti-inflammatory activity of the antibiotic.

The complex etiology of IBD remains to be fully understood, nevertheless during the last years gut commensal microbiota has arisen as an important factor in its pathogenesis. Although the cause-effect relationship is not clear, dysbiosis and aberrant inflammatory responses are common components of IBD. The characteristics of a specific dysbiotic state depend upon the starting commensal microbiota. In the present study, ceco-colonic microbiota in healthy animals was dominated by the phyla Bacteroidetes (48.4%) and Firmicutes (37%); with other archetypical phyla, Verrucomicrobia, Deferribacteres and Proteobacteria, representing less than 10% of the total microbiota, in agreement with previous data and our previous observations.[64–67] With this microbial composition, the animals used in the present study should be classified as belonging to the enterotype 2 (dominated by Bacteroidetes); which corresponds to the human enterotype 1 (enriched in *Bacteroides*).[65,68] Besides this characteristic, other microbial features at lower phylogenetic levels related the animals used to the human enterotype 1. For instance, the relatively high presence of *Parabacteroides* (genus), *Bacteroides* spp. and Porphyromonadaceae (family) are also characteristics of the human enterotype 1.[68] Interestingly, we were able to detect the presence of *Prevotella* (genus) at significant levels. An enrichment in *Prevotella* defines the human enterotype 2.[68] Therefore, it is feasible to assume that the animals used might share characteristics of the human enterotypes 1 and 2, being a hybrid of these two enterotypes. Further studies are needed to ascertain this possibility or the existence of a third murine enterotype, equivalent to the human *Prevotella* enterotype.[65] In any case, this interspecies conserved bacterial composition facilitates the translational validation of preclinical studies using mice.

It is well-established today that intestinal inflammation is associated to a state of dysbiosis. However, numerous and diverse microbial changes have been associated to inflammation in the literature,[23,36,69–77] probably reflecting the complexity and diversity of the microbiota and the multiple factors that influence intestinal microbial ecology. Classically, intestinal inflammation, both in animal models and in IBD in humans, has been associated to a loss of bacterial biodiversity.[3,20,41,65,76,78–82] However, other reports, mainly in animal models, indicate that biodiversity might be maintained during inflammation.[23,24,36,74,83] In our conditions, no loss of biodiversity was observed during DSS-induced colitis, according to the indices of α-diversity calculated or the number of OTUs detected. These observations suggest a redistribution of the existing microbiota during inflammation, rather than the disappearance of existent bacterial groups or the apparition of new ones.[83] This was further supported by the PCoA analysis, which shows a separation between healthy (vehicle-vehicle group) and colitic (vehicle-DSS group) animals, indicating the presence of a dysbiosis during DSS-induced colitis.

DSS-induced colitis-associated dysbiosis was characterized by an increase in the phyla Verrucomicrobia and Deferribacteres, together with a decrease in Bacteroidetes. These changes partially agree with those previously described for DSS-induced colitis in mice. Indeed, several studies showed a similar decrease in Bacteroidetes, both in animals[23,36,59,71,75,76] and in IBD[79,84,85]. Similarly, our observations are in agreement with previous studies showing increases in the abundance of Deferribacterales[74,77] and Verrucomicrobia-related bacterial groups.[73–75,77] In particular, the increase in the relative abundance of Verrucomicrobia was due to a single OTU, OTU03 *Akkermansia*, which represents the majority of the phylum. *Akkermansia* is regarded as a bacterial group adapted to grow in a DSS-rich environment[76] and with high oxigen tolerance, being able to suvive oxidative state observed during inflammation.[3] Therefore, the outgrowth of this group might be a direct consequence of the local environment associated to the use of DSS as inductor of inflammation. The phylum Firmicutes have also been implicated in intestinal inflammation. However, the changes described are not consistent and either increases,[23,86] decreases[87] or a stable abundance[88] have been described. In our conditions, the phylum Firmicutes, *per se*, was not affected during inflammation, probably because it includes multiple genera and the expansion of some of them is compensated by the reduction of others[74]. However, increases in relative abundance were observed for specific OTUs within this phylum, belonging mainly to the clostridia-related groups, in agreement with previous observations.[36,72] Overall, these observations support the presence of a dysbiotic state during inflammation, based largely on the redistribution of the existing bacterial populations. Differences in healthy GCM,

environmental conditions and experimental setting are probably responsible for the large variability observed across studies in the characteristics of inflammation-associated microbiota. Therefore, a unique bacterial fingerprint for colitis-associated dysbiotic microbiota cannot be established.

In normal animals, rifaximin treatment was associated to relatively minor alterations of the microbiota, particularly at the higher dose tested; in agreement with previous studies indicating limited modulatory effects of rifaximin on intestinal microbiota.[19,20,23,24,89,90] Rifaximin-treated animals, irrespective of the dose considered, clustered together with control animals (vehicle-vehicle group) in the PCoA analysis (Fig. 9), indicating a similar bacterial composition. In any case, some changes were observed mainly in animals treated at the dose of 50 mg/kg. Specifically, an increase in Bifidobacteriaceae and Deferribacteres and a decrease in Verrucomicrobiaceae were observed, due to changes in their corresponding genera (*Bifidobacterium, Mucispirillum* and *Akkermansia*, respectively). Moreover, small changes (increases and decreased in relative abundance) in specific OTUs of the phylum Firmicutes were also noticed. The functional significance of these changes is difficult to ascertain. *Bifidobacterium*, with increased relative abundance, is used as a probiotic for its positive immunomodulatory activity, sustaining intestinal homeostasis.[91,92] Therefore, an increase in its relative abundance could be regarded as a protective modulatory effect against inflammation. On the other hand, several clostridia-related OTUs implicated in the suppression of immune responses to self and bacterial antigens, promoting epithelial repairmen and tolerance to microbes,[93,94] showed inconsistent changes (in particular OTU254 *Clostridium_XVIII*, with an increase; and OTUs 24 57 *Clostridium_XIVa*, with a decrease). Therefore, a potential microbial-mediated modulatory role of rifaximin on inflammation cannot be inferred from the present observations.

In colitic animals, treatment with rifaximin did not affect bacterial richness, according to alpha diversity or the number of OTUs detected. When assessing beta diversity, the treatment with rifaximin (irrespective of the dose considered) prevented the separation of these animals and in the PCoA analysis inflamed animals treated with rifaximin clustered together with non-inflamed animals (receiving either vehicle or rifaximin). Confirming this observation, the Adonis analysis revealed a significantly different microbiota composition for rifaximin-DSS-treated vs. vehicle-DDS-treated animals. Moreover, Adonis analysis also showed similarity in the microbiota of the rifaximin-DSS, rifaximin-vehicle and vehicle-vehicle groups, in agreement with the clustering revealed by the PCoA analysis. Nonetheless, specific changes in ceco-colonic microbiota at

different phylogenetic levels were detected in rifaximin-treated colitic animals when compared to the healthy ones. However, no consistency was observed between the two doses of rifaximin tested, probably because of the complexity and variability of the microbiota. The only common change was an increase in the relative abundance of Proteobacteria, affecting several Enterobacteriaceae-related genera and OTUs. This might result from the combined effect rifaximin-DSS, since changes in Proteobacteria were not observed in non-inflamed animals receiving either vehicle or rifaximin.

From these observations, it is feasible to speculate that although having relatively minor effects on microbiota, as discussed above, the changes induced by rifaximin persist during inflammation and prevent a shift in the microbiota towards that observed in colitic animals not receiving the antibiotic. Interestingly, the clinical course of inflammation and the associated structural and molecular alterations were not affected by rifaximin (as previously discussed). Therefore, it seems feasible to dissociate rifaximin-induced microbial changes and inflammation; thus suggesting that, overall, rifaximin-shaped microbiota has no beneficial effects on intestinal inflammation. Accordingly, potential beneficial effects of rifaximin cannot be attributed to its antimicrobial effects at least as it relates to the DSS-induced colitis model in mice. This, contrasts with the reported beneficial effects of rifaximin on other animal models of intestinal inflammation. For instance, rifaximin ameliorated stress-induced gut inflammation in rats through the modulation of ileal microbiota[95] and TNBS-induced colitis in Balb/c mice by reducing bacterial translocation[37], thus emphasizing the importance of model- and species-related differences.

Compared to the limited effects of rifaximin, the treatment with doxycycline in healthy animals was associated to extensive changes in the ceco-colonic microbiota. Although its dysbiotic effect, doxycycline did not affect bacterial richness, in agreement with previous observations in mice.[23–25] Moreover, the PCoA analysis showed a distinct clustering for doxycycline-treated animals, which were completely separated from other experimental groups, further indicating significant differences in their microbiota. This contrast with data from Garrido-Mesa et al. (2108)[23,24] showing that rifaximin- and doxycycline-treated animals had a tendency to group together, indicating a similar dysbiotic effect for both antibiotics. Again, this difference might be related to the original microbiota of the animals used. Interestingly, all animals receiving doxycycline, with or without colitis, were mixed in the same clusters in the PCoA analysis, thus indicating similar bacterial composition. Indeed, comparable microbial changes were observed

in their phylogenetic analysis. Taking into account the protective effects against DSS-induced colitis observed for doxycycline, these observations might suggest a shift towards a protective microbiota during doxycycline-induced dysbiosis. Altogether, these evidences indicate that beneficial effects of doxycycline in colitis are likely to relate to an interaction of the immunomodulatory and the antimicrobial actions of the antibiotic.[23,24]

Although at higher taxonomic levels the architecture of commensal microbiota was similar, doxycycline induced numerous changes at the genus and OTU levels; that were either increased or decreased in abundance, possibly mirroring the sensitivity of individual bacterial groups towards the antibiotic[25] as well as the presence of intra- and inter-taxon competition.[74] At the phylum level, the only significant finding was a reduction in the relative abundance of Verrucomicrobia, associated to a reduction in the only genus (*Akkermansia*) and OTU (OTU03) included; contrasting with previous reports showing no changes in this bacterial group.[23,24] This change is somehow surprising taking into consideration the beneficial effects of doxycycline, since *Akkermansia*-related bacteria are regarded as a component of a healthy microbiota and a depletion in this group has been associated to IBD.[96–98] Bacteroidetes were not affected as a phylum, although a distinct pattern of OTUs that were either increased or decreased in abundance was detected.[25] In particular, an enrichment in OTUs related to the genus *Barnesiella* was detected. *Barnesiella* may confer anti-inflammatory properties in the context of DSS challenge since it correlates positively with lower disease activity indices[99] and it is able to eliminate and protect against the intestinal dominance of antibiotic-resistant pathogenic bacteria.[100] Finally, although the phylum Firmicutes was not affected *per se*, as previously described,[23,25] it included a large number of OTUs with significant changes in their relative abundance (increase or decrease). Overall, the OTUs changed overlap with those also described by Becker et al. (2107).[25] Some changes that might bear interest in the contest of intestinal inflammation include: i) a general reduction in Clostridiales, considered pathogenic groups; ii) an increase in Clostridia cluster XIVa-related OTUs, which promote restoration of intestinal homeostasis;[93,94] iii) an increase in *Lactobacillus*, a well-known probiotic bacterial strain with anti-inflammatory and immunomodulatory properties;[95] iv) an increase in *Ruminococcus*, a commensal bacteria that diminishes intestinal colonization of pathogens.[44] Altogether, it is feasible to speculate that the interaction between the immunomodulatory and antimicrobial effects of doxycycline generates a local anti-inflammatory environment that results in the improvement of DSS-induced colitis.

Although out of the scope of the present work, given the positive results observed for doxycycline, we did try to relate its anti-inflammatory and antimicrobial activities. For this, we assessed associations between clinical signs and immune-related markers and OTUs significantly modified by the treatment with the antibiotic (see Fig. 23). This analysis identified several OTUs, belonging essentially to the phylum Firmicutes, correlating positively and negatively with mainly immune-related markers (TNFα, IL-6 and RegIIIγ) and macroscopic inflammatory scores. Further follow-up studies are needed to ascertain with precision the bacterial(s) strain(s) driving these effects and, therefore, with the potential to be used as probiotic for the treatment of intestinal inflammation.

In summary, our results show that, in the DSS-induced colitis model in mice, rifaximin does not exhibit significant immunomodulatory nor microbial-modulatory effects consistent with an anti-inflammatory activity. The negative findings of this study does not exclude an immunomodulatory and/or microbial-modulatory action of rifaximin in humans, thus explaining its effectiveness in IBS and IBD. Moreover, taking into account the, at least partially, postulated mechanism of action of rifaximin through human PXR receptors, the DSS-induced colitis model in mice might not be adequate to fully address the mechanisms of action of rifaximin modulating intestinal inflammation. On the other hand, doxycycline, in the same colitis model, showed positive anti-inflammatory effects and induced an, apparently, protective dysbiotic state.

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General discussion

Compelling evidences indicate that the microbiota plays a significant role in the maintenance of the GI homeostasis. Moreover, a normal microbiota seems to be necessary to develop and maintain a mature and competent immune system, both inside and outside the GI tract.[81] In this sense, states of dysbiosis have been associated to several pathophysiological conditions affecting the digestive system, in particular inflammatory and functional gastrointestinal.[7,15] In these conditions, the combination of an altered immune-related state and a dysregulated microbiota might lead to abnormal immune responses and the development of sustained states of inflammation and tissue damage.[29,78] Because of this double immunological/microbial component, antibiotics with immunomodulatory properties have been suggested as a possible therapeutic approach for IBD and IBS.[102,104] In this sense, rifaximin is a wide-spectrum antibiotic, with double antimicrobial and immunomodulatory activity, effective in the treatment of several GI conditions implicating states of dysbiosis, including IBD and IBD. However, the exact mechanisms of action remain unclear.[14,17,18] Therefore, in this work, we explored the effects of rifaximin on normal GCM in mice as well as its potential immunomodulatory and microbial-modulatory effects in states of intestinal inflammation.

Rifaximin has limited immunomodulatory and antimicrobial effects on normal GCM in mice

In otherwise healthy mice, biodiversity and composition of the luminal ceco-colonic microbiota was essentially not affected by rifaximin, despite the antimicrobial effects observed in *in vitro* conditions. These observations agree with previous studies in humans and mice, showing a poor antimicrobial activity for rifaximin when used *in vivo*.[118,152] Similarly, bacterial adherence to the colonic wall was not affected by the antibiotic. Taking into account these effects, some authors describe the microbial actions of rifaximin as "eubiotic", suggesting that the antibiotic, unlike other antimicrobial agents, would act as a microbial modulator preventing an abnormal bacterial colonization and favoring the restoration of a normal microbiota during a state of dysbiosis.[7,116,117] During the phylogenetic analysis, these limited modulatory effects of rifaximin were evidenced by changes (either increases or decreases in relative abundance) in specific OTUs distributed among the main phyla detected. The biological significance of these changes is difficult to establish. Nevertheless, the weak microbial-modulatory activity observed in normal conditions do not exclude more pronounced effects during a state of dysbiosis.

Moreover, rifaximin did not induce changes consistent with the induction of an inflammatory-like state within the colon. Changes in immune-related markers consistent with an activation of the immune system have been reported for other antibiotics eliciting significant changes in the GCM.[41,153,154] In this sense, the lack of immunomodulatory effects of rifaximin in normal conditions agrees with the marginal effects observed on GCM.[106,108,115,152,155,156] Therefore, it is feasible to speculate that rifaximin does not alter intestinal homeostasis, maintaining a "eubiotic" state without consequences in the local immune activity.[108,152] Further supporting the view that rifaximin maintains gastrointestinal homeostasis is the fact that host-bacterial interaction systems, at least as it relates to antimicrobial peptides and TLR2, 3, 4, 5 and 7, were essentially not affected by the antibiotic. Again, the lack of immunomodulatory activity might be attributed to the physiological state in which the compound was tested, not excluding other effects under pathophysiological conditions.

Administration of DSS leads to a dysbiotic state with an overt colonic inflammation and up-regulated expression of immune-related markers

Exposure to DSS led to the development of a colitic state characterized by the presence of clinical signs, local (colonic) macroscopic and microscopic (histopathological) alterations and molecular (gene expression) changes, similar to that previously published for this model;[132,133,135,140] and reminiscent to the pathophysiological changes observed in IBD patients[135].

As mentioned, intestinal inflammation has been associated to microbial dysbiosis, although the casual relationship is not clear.[7] Accordingly, in animals with DSS-induced colitis, a state of dysbiosis was detected in the ceco-colonic microbiota. Despite the dysbiotic state, a maintenance of biodiversity, as determined by different indexes of alpha diversity or according to the number of OTUs detected in the phylogenetic analysis, was observed. This finding agrees with previous reports showing no changes in biodiversity during intestinal inflammation,[42,140,148] but contrasts with studies showing a loss in microbial diversity and richness during intestinal inflammation.[7,15,30,88,89,105,108,142,143,145,157,158] This variability might be related to the experimental model and the species, as well as to the origin of the samples used for microbial characterization (tissue vs. fecal content).[140] The maintenance of biodiversity observed suggest that, during colitis, rather than a loss of bacterial richness, there is a redistribution of the existing bacteria.[42] This was confirmed by the differences observed in beta-diversity (PCoA analysis), showing

separate clustering for healthy vs. colitic mice, and by the changes detected in the phylogenetic analysis of the ceco-colonic microbiota, especially at the OTU level. A few studies have attempted the characterization of the microbiota during DSS-induced colitis in mice.[140,142,143,146–148] Overall, these studies, as well as the present results, confirm the presence of a dysbiotic state, although with relatively high variability in the bacterial groups affected. This probably reflects the complexity of the microbiota, as well as differences in healthy GCM, environmental conditions and experimental setting.[28,30,159] Therefore, it seems feasible to accept that a unique bacterial fingerprint for colitis-associated dysbiotic microbiota cannot be established.

In the present experimental conditions, DSS-induced colitis-associated dysbiosis was characterized by a depletion in Bacteroidetes and an enrichment in Deferribacteres, Verrucomicrobia and Clostridiales (Lachnospiraceae and unclassified Clostridiales). It is difficult to speculate about the biological significance of these changes since the taxonomic level at which microbiota analysis becomes biologically meaningful is not defined and it is unclear how important within-taxa variations are to higher-level microbial taxa.[148] Indeed, in many cases, bacterial differences and dynamics at the OTU level observed in our study were hidden when relative bacterial abundances were considered at higher taxonomic levels. Therefore, our results agree with Berry et al. (2012),[148] indicating that analysis at higher taxonomic levels is not enough to reveal important colitis-associated microbiota shifts.

Rifaximin does not exhibit anti-inflammatory activity in mice with DSS-induced colitis

Beneficial effects of rifaximin in humans have been related, at least partially, to its ability to modulate the immune response.[105,109,160] However, in the present conditions, rifaximin did not exhibit anti-inflammatory nor positive immunomodulatory effects during DSS-induced colitis in mice. Indeed, preventive treatment with rifaximin did not modify the clinical course of DSS-induce colitis, nor the colonic macroscopical and microscopical (histopathological) alterations associated to the process. Moreover, the antibiotic exacerbated the up-regulated gene expression observed for some immune-related markers, thus indicating a tendency to worsen the inflammatory reaction. These observations agree with published data in the same model, showing that rifaximin was unable to reduce, and in some cases even increased, colonic expression of pro-inflammatory cytokines.[106,118] However, positive (anti-inflammatory) modulatory effects have been described for rifaximin during DNBS- and TNBS-induced colitis

in mice,[156,161] thus suggesting model-related differences in the mechanism of action of the antibiotic.

Some reports indicate that the positive immunomodulatory effects of rifaximin observed in humans are PXR-mediated. In fact, rifaximin is a selective agonist of the human PXR and seems to up-regulate the expression of PXRs.[105,118,162] In the present conditions, colonic expression of PXRs had a clear tendency towards the down-regulation in colitic animals, and rifaximin did not affect such change. Lack of PXR-mediated effects might explain the negative results obtained. However, the positive effects observed in other murine models of colitis[156,161] suggest other, PXR-independent, mechanisms mediating the immunomodulatory and anti-inflammatory effects of rifaximin.[116,161,163]

Rifaximin has limited modulatory effects on ceco-colonic microbiota during DSS-induced colitis

In animals with colitis and treated with rifaximin, microbial composition, although with some changes, was close to that observed in healthy animals, with or without rifaximin. Firstly, there were no changes in alpha diversity or in the number of OTUs detected. Secondly, beta-diversity (PCoA) showed proximity between healthy, with or without rifaximin, and rifaximin-treated colitic animals; thus indicating a similar microbial composition; different to that observed in non-treated colitic mice, which formed a clear separated cluster. Rifaximin-mediated selective modulation of the microbiota, without altering the overall microbial composition but promoting beneficial bacterial strains (such a *Bifidobacteria*, *Bifidobacterium* and *Faecalibacterium prausnitzii*) has been suggested as a part of the underlying mechanism mediating the beneficial effects observed in IBD.[108,117,152] In line with these observations, the minor modulatory effects associated to rifaximin in the present conditions, as discussed above, might be sufficient to prevent a complete shift in the microbiota towards that of an inflamed state but are insufficient to modulate (prevent) inflammation and/or immune activation through microbial-dependent mechanisms. This can also be related to the minor changes induced by rifaximin on host-microbial interaction systems, at least as it relates to the local expression of TLRs. Under these conditions, microbial signaling to the host might be essentially the same to that associated to a normal, healthy, GCM.

Preventive treatment with doxycycline attenuates clinical signs of colitis, has an anti-inflammatory-like activity and induces an, apparently, protective dysbiotic state

Doxycycline is an immunomodulatory antibiotic with proven beneficial effects in experimental colitis.[106,126,156] For this reason, it was included as a positive control in the present studies.

Administration of doxycycline in normal animals led to a dysbiosis without evidences of local immune activation or the induction of an inflammatory-like state within the colon. Doxycycline-induced dysbiosis was associated to a microbiota significantly different to that observed in healthy animals treated with rifaximin administration; according to alpha and beta diversities and the number of OTUs detected. Consistent with this, a small loss of biodiversity and extensive changes at different phylogenetic levels were observed in, otherwise healthy, doxycycline-treated animals when compared with non-treated healthy animals. Interestingly, similar dysbiotic state was observed in colitic mice treated with doxycycline, which clustered together with doxycycline-treated healthy mice in the PCoA. Moreover, in these animals, an attenuation of the inflammatory state and a general positive immunomodulatory effect was observed. It is difficult to stablish a direct causal relationship between the microbial changes observed and the attenuation of the colitic state observed in these animals. Nevertheless, it is feasible to speculate that doxycycline-induced dysbiosis might result in a beneficial/protective microbiota, which, at least partially, might contribute to the amelioration of the colitic state. In this sense, doxycycline induced a relative enrichment in bacterial groups regarded as beneficial (i.e. OTUs related to the genus *Barnesiella*, Clostridia cluster XIVa-related OTUs, *Lactobacillus* or *Ruminococcus*) and a simultaneous reduction in groups regarded as pathogenic (i.e. a general reduction in Clostridiales), thus supporting a potential microbial-derived beneficial effect. These observation warrant further studies addressing in details the microbial modulatory effects of doxycycline and directed towards the identification of specific bacterial(s) group(s) responsible of the positive effects observed here and, therefore, with the potential to be developed as probiotics to treat intestinal inflammation.

Overall, the present results show that, in the DSS-induced colitis model in mice, rifaximin does not exhibit significant immunomodulatory nor microbial-modulatory effects. Nevertheless, the negative findings of this study does not exclude an immunomodulatory and/or microbial-modulatory action of rifaximin in humans, thus explaining its effectiveness in IBS and IBD.

Taking into account the, at least partially, postulated mechanism of action of rifaximin through human PXR receptors, the DSS-induced colitis model in mice might not be adequate to fully address the mechanisms of action of rifaximin modulating intestinal inflammation.

Conclusions

1. In normal mice, rifaximin does not affect neither luminal ceco-colonic commensal microbiota, as indicated by a maintenance of alpha and beta diversity, nor bacterial wall adherence to the colonic wall.

2. In normal mice, rifaximin does not induce neither macroscopic nor microscopic changes consistent with the induction of a colonic inflammatory-like state.

3. In normal mice, rifaximin does not affect the colonic expression of immune–related markers, at least as it relates to pro- and anti-inflammatory cytokines, nor antimicrobial peptides, nor the pregnane X receptor, nor Toll-like receptors 2, 5 and 7; but induces a transitory, dose-related, up-regulation of Toll-like receptors 3 and 4.

4. In the model of dextran sulfate sodium (DSS)-induced colitis in mice, a dysbiotic state with a variation in beta diversity, but not alfa diversity, is observed. This state of dysbiosis is characterized, at the phylum level, by an increase in the relative abundance of Verrucomicrobia and Deferribacteres and a decrease in the relative abundance of Bacteroidetes. At the OTU level, these changes correspond to an increase in the relative abundance of OTU03 Verrucomicrobiaceae-*Akkermansia*, a decrease in the relative abundance of OTU21 Bacteroidaceae-*Bacteroides* and a general increase in the relative abundance of OTUs corresponding to the genus unclassified_Lachnospiraceae.

5. In the model of dextran sulfate sodium (DSS)-induced colitis in mice, preventive treatment with rifaximin does not modify the clinical course of inflammation nor the associated macroscopic and microscopic changes within the colon. Rifaximin exacerbates colitis-associated up-regulation of inflammatory markers and down-regulates the expression of the pregnane X receptor. A simultaneous modulation of colonic Toll-like receptors-dependent host-bacterial interaction systems is also present, with a down-regulation of Toll-like receptors 3, 4 and 5, and no changes in the expression of Toll-like receptors 2 and 7. Likewise, in this model, rifaximin has no effects on the colonic expression of antimicrobial peptides.

6. In the model of dextran sulfate sodium (DSS)-induced colitis in mice, preventive treatment with rifaximin does not affect luminal ceco-colonic commensal microbiota. In these conditions, alpha diversity indices, beta diversity, as shown by the Principal

Coordinate of Analysis, and relative bacterial abundance, at either the phylum, family, genus or OTU levels, are equivalent to that determined in normal mice, with or without rifaximin administration.

7. In normal mice, doxycycline induces a dysbiotic state characterized by a variation in beta, but not alpha, diversity. This dysbiotic state is characterized by extensive changes at the genus and OTU levels; corresponding to an increase in relative bacterial abundance within the families Bacteroidaceae and Ruminococcaceae; and a decrease in relative bacterial abundances within the families Porphyromonadaceae, Verrucomicrobiaceae, Lachnospiraceae and unclassified_Clostridiales.

8. In the model of dextran sulfate sodium (DSS)-induced colitis in mice, preventive treatment with doxycycline attenuates the clinical course of inflammation but not the associated macroscopic and microscopic changes within the colon. At the molecular level, doxycycline normalizes the expression of pro-inflammatory cytokines and the antimicrobial peptide Regenerating islet-derived protein 3 gamma (RegIIIγ), without significant effects in the expression of Toll-like receptors, the antimicrobial peptides defensin alpha 24 (Defα24) and resistin-like molecule beta (RELMβ) and the pregnane X receptor.

9. Ceco-colonic microbial composition in doxycycline-treated animals with dextran sulfate sodium (DSS)-induced colitis is similar, as it relates to alpha and beta diversity, to that observed in doxycycline-treated animals without inflammation. Nonetheless, significant changes in bacterial relative abundance within the phylum Firmicutes, at the family, genus and OTU levels, are observed during colitis.

10. The model of dextran sulfate sodium (DSS)-induced colitis in mice does not reproduce the positive effects observed for rifaximin in a clinical set-up, applied to the treatment of inflammatory and functional gastrointestinal disorders, and, therefore, cannot be regarded as an appropriate animal model to study the mechanisms of action of rifaximin in humans.

Conclusiones

1. En ratones normales, la rifaximina no afecta ni a la microbiota comensal ceco-cólica luminal, tal y como indica el mantenimiento de las diversidades de tipo alfa y beta, ni a la adherencia bacteriana a la pared del colon.

2. En ratones normales, la rifaximina no induce cambios macroscópicos ni microscópicos compatibles con la presencia de una activación inmune o la inducción de un estado de tipo inflamatorio en el colon.

3. En ratones normales, la rifaximina no afecta la expresión cólica de marcadores inmunológicos, al menos en lo que se refiere a citoquinas pro- y anti-inflamatorias, de péptidos antimicrobianos, del receptor X de pregnano o de los receptores de tipo Toll 2, 5 y 7; pero induce una regulación transitoria, dosis-dependiente, al alza de los receptores de tipo Toll 3 y 4.

4. En el modelo de colitis inducida por dextrano sulfato sódico (DSS) en ratones, se observa un estado disbiótico con un cambio en la diversidad de tipo beta, pero no en la de tipo alfa. Este estado de disbiosis se caracteriza, a nivel de filo, por un aumento en la abundancia relativa de Verrucomicrobia y Deferribacteres y una disminución en la abundancia relativa de Bacteroidetes. A nivel de OTU, estos cambios se corresponden con un aumento en la abundancia relativa de la OTU03 Verrucomicrobiaceae-*Akkermansia*, una disminución en la abundancia relativa de la OTU21 Bacteroidaceae-*Bacteroides* y un aumento general en la abundancia relativa de OTUs correspondientes a la familia Lachnospiraceae.

5. En el modelo de colitis inducida por dextrano sulfato sódico (DSS) en ratones, el tratamiento preventivo con rifaximina no modifica el curso clínico de la inflamación ni los cambios macroscópicos y microscópicos observados a nivel del colon. La rifaximina exacerba la regulación al alza de los marcadores inflamatorios asociados a la colitis y regula a la baja la expresión del receptor X de pregnano. Simultáneamente, se observa una modulación de los sistemas de interacción hospedador-microbiota dependientes de receptores de tipo Toll, con una regulación a la baja de los receptores de tipo 3, 4 y 5, y sin cambios en la expresión de los receptores de tipo 2 y 7. Asimismo, en este modelo, la rifaximina no tiene efectos sobre la expresión cólica de péptidos antimicrobianos.

6. En el modelo de colitis inducida por dextrano sulfato sódico (DSS) en ratones, el tratamiento preventivo con rifaximina no afecta la microbiota comensal ceco-cólica luminal. En estas condiciones, la diversidad tipo alfa, la diversidad tipo beta, tal como muestra el análisis de coordenadas principales, y la abundancia bacteriana relativa, ya sea a nivel de filo, familia, género u OTU, es equiparable a la determinada en ratones normales, con o sin administración de rifaximina.

7. En ratones normales, el tratamiento con doxiciclina induce un estado disbiótico caracterizado por un cambio en la diversidad de tipo beta, pero no en la de tipo alfa. Este estado disbiótico se caracteriza por cambios extensos a nivel de OTU y de género; correspondientes a un aumento de la abundancia bacteriana relativa dentro de las familias Bacteroidaceae y Ruminococcaceae; y una disminución de la abundancia bacteriana relativa dentro de las familias Porphyromonadaceae, Verrucomicrobiaceae, Lachnospiraceae y el grupo de los Clostridiales.

8. En el modelo de colitis inducida por dextrano sulfato sódico (DSS) en ratones, el tratamiento preventivo con doxiciclina atenúa el curso clínico de la inflamación, pero no los cambios macroscópicos y microscópicos asociados observados en el colon. A nivel molecular, la doxiciclina normaliza la expresión de citoquinas pro-inflamatorias y del péptido antimicrobiano proteína regeneradora de islote 3-gamma (RegIIIγ), sin efectos significativos en la expresión de los receptores de tipo Toll, los péptidos antimicrobianos defensina alfa 24 (Defα24) y resistina de tipo beta (RELMβ) y el receptor X de pregnano.

9. La microbiota luminal ceco-cólica en animales tratados con doxiciclina con colitis inducida por dextrano sulfato sódico (DSS) es similar, en lo que se refiere a las diversidades de tipo alfa y beta, a la observada en animales tratados con doxiciclina sin inflamación. No obstante, durante la colitis se observan cambios significativos en la abundancia relativa de bacterias dentro del filo Firmicutes, a nivel tanto de familia, como de género y de OTU.

10. El modelo de colitis inducida por dextrano sulfato sódico (DSS) en ratones no reproduce los efectos positivos observados para la rifaximina en un escenario clínico, aplicada al tratamiento de trastornos gastrointestinales inflamatorios y funcionales, y,

por lo tanto, no se puede considerar un modelo animal apropiado para estudiar los mecanismos de acción de la rifaximina en humanos.

Conclusions

1. En ratolins normals, la rifaximina no afecta ni la microbiota comensal ceco-còlica luminal, tal com indica el manteniment de les diversitats de tipus alfa i beta, ni l'adherència bacteriana a la paret del còlon.

2. En ratolins normals, la rifaximina no indueix canvis macroscòpics ni microscòpics compatibles amb la presència d'una activació immune o la inducció d'un estat de tipus inflamatori en el còlon.

3. En ratolins normals, la rifaximina no afecta l'expressió còlica de marcadors immunològics, almenys pel que fa a citocines pro- i anti-inflamatòries, de pèptids antimicrobians, del receptor X de pregnà o dels receptors de tipus Toll 2, 5 i 7; però indueix una regulació transitòria, dosi-depenent, a l'alça dels receptors de tipus Toll 3 i 4.

4. En el model de colitis induïda per dextrà sulfat sòdic (DSS) en ratolins, s'observa un estat disbiòtic amb un canvi en la diversitat de tipus beta, però no en la de tipus alfa. Aquest estat de disbiosi es caracteritza, a nivell de fílum, per un augment en l'abundància relativa de Verrucomicrobia i Deferribacteres i una disminució en l'abundància relativa de Bacteroidetes. A nivell d'OTU, aquests canvis es corresponen amb un augment en l'abundància relativa de l'OTU03 Verrucomicrobiaceae-*Akkermansia*, una disminució en l'abundància relativa de l'OTU21 Bacteroidaceae-*Bacteroides* i un augment general en l'abundància relativa d'OTUs corresponents a la família Lachnospiraceae.

5. En el model de colitis induïda per dextrà sulfat sòdic (DSS) en ratolins, el tractament preventiu amb rifaximina no modifica el curs clínic de la inflamació ni els canvis macroscòpics o microscòpics observats a nivell del còlon. La rifaximina exacerba la regulació a l'alça dels marcadors inflamatoris associats a la colitis i regula a la baixa l'expressió del receptor X de pregnà. Simultàniament, s'observa una modulació dels sistemes d'interacció hoste-microbiota dependents de receptors de tipus Toll; amb una regulació a la baixa dels receptors de tipus 3, 4 i 5, però sense canvis en l'expressió dels receptors de tipus 2 i 7. Així mateix, en aquest model, la rifaximina no té efectes sobre l'expressió còlica de pèptids antimicrobians.

6. En el model de colitis induïda per dextrà sulfat sòdic (DSS) en ratolins, el tractament preventiu amb rifaximina no afecta la microbiota comensal ceco-còlica luminal. En aquestes condicions, la diversitat tipus alfa, la diversitat tipus beta, tal com mostra l'anàlisi de coordenades principals, i l'abundància bacteriana relativa, ja sigui a nivell de fílum, família, gènere o OTU, és equiparable a la determinada en ratolins normals, amb o sense administració de rifaximina.

7. En ratolins normals, el tractament amb doxiciclina indueix un estat disbiòtic caracteritzat per un canvi en la diversitat tipus beta, però no en la diversitat tipus alfa. Aquest estat disbiòtic es caracteritza per amplis canvis a nivell d'OTU i de gènere; corresponents a un augment en l'abundància bacteriana relativa dins de les famílies Bacteroidaceae i Ruminococcaceae; i una disminució de l'abundància bacteriana relativa dins de les famílies Porphyromonadaceae, Verrucomicrobiaceae, Lachnospiraceae i el grup dels Clostridials.

8. En el model de colitis induïda per dextrà sulfat sòdic (DSS) en ratolins, el tractament preventiu amb doxiciclina atenua el curs clínic de la inflamació, però no els canvis macroscòpics o microscòpics associats observats en el còlon. A nivell molecular, la doxiciclina normalitza l'expressió de citocines pro-inflamatòries i del pèptid antimicrobià proteïna regeneradora d'illot 3-gamma (RegIIIγ), sense efectes significatius en l'expressió dels receptors de tipus Toll, els pèptids antimicrobians defensina alfa 24 (Defα24) i resistina de tipus beta (RELMβ) i el receptor X de pregnà.

9. La microbiota luminal ceco-còlica en animals tractats amb doxiciclina amb colitis induïda per dextrà sulfat sòdic (DSS) és similar, pel que fa a les diversitats tipus alfa i beta, a l'observada en animals tractats amb doxiciclina sense inflamació. No obstant això, durant la colitis s'observen canvis significatius en l'abundància relativa de bacteris dins del fílum Firmicutes, a nivell tant de família, com de gènere i d'OTU.

10. El model de colitis induïda per dextrà sulfat sòdic (DSS) en ratolins no reprodueix els efectes positius observats per a la rifaximina en un escenari clínic, aplicada al tractament de trastorns gastrointestinals inflamatoris i funcionals, i, per tant, no es pot considerar un model animal apropiat per estudiar els mecanismes d'acció de la rifaximina en humans.

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Appendices

Publications derived from this Thesis

Papers:

M. Ferrer, V. Martínez. Effects of Rifaximin on Luminal and Wall-Adhered Gut Commensal Microbiota in Mice. *Microbial Ecology* (submitted).

Abstracts:

M. Ferrer, V. Martínez. Rifaximin-dependent modulation of gut commensal microbiota and host:bacterial interactions in a murine model of DSS-induced colitis. *Acta Physiologica* (2019) 227 (S718): 46.

Federation of European Physiological Societies (FEPS). Bologna – Italy, September 10-13, 2019. Oral communication.

M. Ferrer, V. Martínez. Modulatory effects of rifaximin on innate immune parameters on a murine model of Dextran Sulfate Sodium (DSS)-induced colitis. *J Physiol Biochem* (2018) 74 (Suppl 1): S93.

XXXIX Congreso de la Sociedad Española de Fisiología (SECF). Cádiz – Spain, September 18-21, 2018. Poster.

M. Ferrer, V. Martínez. Modulation of inflammation and local innate immune components by rifaximin in a murine model of DSS-induced colitis.

Toll 2018 Editing Innate Immunity. Porto – Portugal, June 6-9, 2018. Poster.

M. Ferrer, M. Aguilera, J. Estévez, V. Martínez. Effects of rifaximin on gut commensal microbiota, Toll-like receptors (TLRs) and inflammatory markers in mice.

Toll 2015 Targeting Innate Immunity. Marbella – Spain, September 30 – October 3, 2015. Poster.

M. Ferrer, M. Aguilera, J. Estévez, P. Vergara, V. Martínez. Effects of rifaximin-α modulating luminal and wall-adhered gut commensal microbiota and Toll-like receptors (TLRs) expression in mice. *Gastroenterology* 2013, 144 (Sppl 1): S-881.

Digestive Disease Week (DDW). Orlando, Florida – USA, 2013, May 18-21, 2013. Poster.

Curriculum vitae

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Education

Oct 2012 – Oct 2015	Universitat Autònoma de Barcelona, Bellaterra, Spain Master's degree in Laboratory Animal Science & Welfare
Sept 2012	Universitat Autònoma de Barcelona, Bellaterra, Spain Course in Training Research Staff Users of Animals for Experimentation and other Scientific Ends
Sept 2011 – Sept 2012	Universitat Autònoma de Barcelona, Bellaterra, Spain Master's degree in Pharmacology
Sept 2004 – Feb 2010	Universitat Autònoma de Barcelona, Bellaterra, Spain Doctor in Veterinary Medicine (DVM)

Work experience

Sept 2017 – Present	Designated Veterinarian Lab Animal Service Vall d'Hebron Research Institute (VHIR) Barcelona
Sept 2013 – Present	PhD student Neuroscience Doctoral Programme Neuroscience Institute Universitat Autònoma de Barcelona, Bellaterra, Spain
May 2012 – Sept 2016	Resident of the European College of Laboratory Animal Medicine (ECLAM) Dept. of Cell Biology, Physiology and Immunology Universitat Autònoma de Barcelona, Bellaterra, Spain

Grants and awards

- Travel award for young scientists - Toll 2018 Editing Innate Immunity congress, Porto – Portugal, June 6-9, 2018.

- Travel award for PhD students, Neuroscience Institute (INc), Universitat Autònoma de Barcelona, Bellaterra, Spain. 2018.

Teaching experience

- Theoretical and practical classes of Laboratory Animal Science course, Veterinary degree, Universitat Autònoma de Barcelona, Bellaterra, Spain.

- Theoretical and practical classes of Course in Training Research Staff Users of Animals for Experimentation and other Scientific Ends, Universitat Autònoma de Barcelona, Bellaterra, Spain.

- Theoretical and practical classes of Master's degree in Laboratory Animal Science & Welfare, Universitat Autònoma de Barcelona, Bellaterra, Spain.

- Co-tutor in Argo Program (approaching science to high school students).

Participation in congresses and meetings

8 abstracts since 2012

8 National congresses

4 International congresses