Universitat Autònoma de Barcelona

Role of Gut Commensal Microbiota Regulating Colonic Sensory-Related Systems

By

Mònica Aguilera Pujabet

A dissertation in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Neuroscience Doctoral Programme

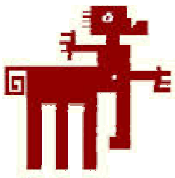
Department of Cell Biology, Physiology and Immunology

Neuroscience Institute

Universitat Autònoma de Barcelona

Advisor

Dr. Vicente Martínez Perea



Bellaterra, Barcelona
2014

Universitat Autònoma de Barcelona

VICENTE MARTÍNEZ PEREA,

Associate Professor of Physiology at the Department of Cell Biology, Physiology and Immunology; Universitat Autònoma de Barcelona.

I hereby certify that the thesis entitled "*Role of gut commensal microbiota regulating colonic sensory-related systems*", submitted by **MÒNICA AGUILERA PUJABET** in partial fulfillment of the requirements for the degree of Doctor of Philosophy, was carried out under my supervision and I authorize the submission to undertake its oral defense.

In witness whereof, I hereby sign this document.

Bellaterra, Barcelona, September 2014

Vicente Martínez Perea, DVM, PhD
Ph.D. Advisor

This work has been funded by the Spanish “Ministerio de Ciencia e Innovación”, project BFU2009-08229 and the FPI program (reference BES-2010-037699; personal support to Mònica Aguilera Pujabet).

Experience is simply the name we give our mistakes

Oscar Wilde (1854 – 1900)
Irish writer and poet

There's Plenty of Room at the Bottom

Richard P. Feynman (1918 – 1988)
American theoretical physicist

AGRAÏMENTS

Han passat 5 anys des de que vaig entrar el departament i no me n'he adonat de com ha passat el temps. Hi ha hagut estones de tot però m'emporto un molt bon record i molta experiència guanyada, en tots el sentits.

En primer lloc, voldria agrair al Vicente, el meu director de tesi, l'ajuda donada i la confiança que ha anat dipositant en mi al llarg del temps. He après molt al teu costat i estic molt contenta de què hagis estat tu el meu director de tesi. El treball és en gran part mèrit teu. Gràcies.

A la Patri, pel seminari de fisiologia de segon de carrera parlant dels ratolins, tenies raó, i per haver-me introduït al departament i al món de l'animal de laboratori.

A Silvia Melgar, por tu amabilidad, ayuda, enseñanza y confiança. La estància en Cork fué más agradable en parte gracias a ti. Tack!

No voldria deixar de mencionar al personal del Fisiologia de la Facultat de Veterinària, ja que en menor o major mesura, també heu contribuït en aquesta tesi. Als professors Ester, Marcel i Maite. No vull oblidar els tècnics i administratius Antonio i David, pels riures (no cal que diguem gràcies a qui... ☺), l'ajuda i la bona cara en tot aquest temps. La següent persona, i no podria ser una altra, és l'Emma. Gràcies pels riures, consells, ajuda i confidències i també pel "*¿qué haces?*" *e*stà clar! I pels riures que ens has causat, que no són pocs.

I ara ja venen el becaris/residents. En general em coneixeu bastant i ja sabeu què penso, com ho penso i perquè ho penso. No caldria que ho deixés per escrit, però com a bona tradició, què menys que deixar plasmades quatre paraules d'agraïment. Cronològicament, gràcies Sandra, sempre seràs la compi de FISH i per l'amistat trobada. Al Joan Antoni perquè sempre seràs el meu "sènior" preferit i per l'ajuda em vas donar des del principi i que has mantingut. A l'Elena Tàpia, por lo compartido dentro y fuera de fisio y lo que queda. Al Joan Burgueño, per la sinceritat, l'ajuda i les converses al 127. Fes-me cas en alguna cosa! Al Sergio, perquè ets un tros de pa i per les converses del despatx que tinc més presents del que et penses. Al Javi, per ser el meu "júnior", per l'ajuda donada, les converses de despatx i perquè espero que em facis cas i no siguis tant pessimista! A la Míriam, per ser companya de foscor del 143, cosa que ha ajudat a descobrir una amistat. A la Marina, perquè ets de les persones que més em coneixen i sempre seràs la meva CAP. Confia en tu. A la Noe, perquè ets un sol i transmets més del què tu creus. I , finalment, tot i

haver treballat o coincidit menys, no voldria deixar de mencionar al Paco, l'Eva, l'Elena Eyre, la Claudia, la Diana, el Jakub i l'Asun.

Finalment, vull agrair a la meva família, i sobretot a la Montse i al Marc. Ja sabeu que sou pilars de la meva vida. Gràcies per aguantar-me, estar allà i fer-me feliç.

Mònica Aguilera Pujabet

Bellaterra, Setembre de 2014

TABLE OF CONTENTS

ABBREVIATIONS

5-HT - serotonin

AMP - Antimicrobial peptide

CB1/2 - Cannabinoid receptor type 1 or 2

cDNA – complementary DNA

CGRP – calcitonin gene related peptide

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

ECs - enterochromaffin cells

ELISA - Enzyme-Linked ImmunoSorbent Assay

FGD - Functional gastrointestinal disorder

FISH - Fluorescent in situ hybridization

GCM - Gut commensal microbiota

GI – Gastrointestinal

IBD – Inflammatory Bowel Disease

IBS - Irritable Bowel Syndrome

IECs – Intestinal epitelial cells

IL-6/10/12p40 -interleukin 6/10/12p40

Itgα/β – Integrin α/β

MMCP-I - mouse mast cell protease I

MOR - mu-opioid receptor (μ opioid receptor)

NGF - Nerve growth factor

PAR-2 – protease activated receptor type 2

PPR - Pattern recognition receptor

Reg3γ - Regenerating islet-derived protein 3 gamma

RELMβ - Resistin-Like Molecule-beta

RIA - Radioimmunoassay

RT-qPCR - Reverse transcription quantitative polymerase chain reaction

SFB - Segmented filamentous bacteria

s-IgA - Secretory IgA

TLR - Toll-like receptor

TNFα - tumor necrosis factor α

TPH 1/2 - Tryptophan hydroxylase isoforms 1 or 2

TRPV1/3/4 - Transient Receptor Potential Vanilloid types 1, 3 or 4

WAS - water avoidance stress

SUMMARY

Gut commensal microbiota (GCM) is a key component of gastrointestinal homeostasis. Functional gastrointestinal disorders (mainly irritable bowel syndrome, IBS) and inflammatory bowel diseases (IBD) have been related to states of altered GCM (dysbiosis). Simultaneously, IBS and IBD patients show local states of abnormal immune activation with altered motor and sensory responses. In particular, in IBS patients sensory alterations lead to characteristic states of visceral hypersensitivity. The exact causal role of GCM remains unclear, but the presence of dysbiosis and the positive effects of antibiotics and some probiotics suggest a key role for the microbiota.

The present work explores the potential role of gut microbiota affecting visceral pain-related sensory systems within the gut and the effects on nociceptive responses. For this purpose, states of real (spontaneous adaptive microbial changes, antibiotic treatment-derived microbial changes) or simulated (direct stimulation of host-bacterial interaction systems) colonic dysbiosis were generated in rats and mice. Colonic microbiota (luminal and wall-adhered) was characterized by fluorescent in situ hybridization (FISH) and qPCR. The immune status of the colon and bacterial-host interactions were determined assessing the expression (RT-qPCR) of pro- and anti-inflammatory cytokines; antimicrobial peptides, integrins and Toll-like receptors (TLRs), the production of secretory IgA (s-IgA), the presence of histopathological alterations and the state of the mucous barrier. Simultaneously, changes in sensory related markers were also assessed. Changes in viscerosensitivity were determined in conscious mice using the Writhing test or following the intracolonic administration of capsaicin.

Overall, antibiotics-induced alterations of the GCM, but not spontaneous changes associated to environmental adaptation, generated a state of local immune activation within the colon. This state was characterized by selective up- and down-regulation of pro- and anti-inflammatory cytokines and host-bacterial interaction markers and changes in the amounts of s-IgA. Similar immune response was observed when a dysbiotic state was simulated in rats by the direct stimulation of colonic TLR4 with bacterial lipopolysaccharides (LPS) or TLR7 with the selective agonist imiquimod. Although these changes, and regardless the model considered, no macroscopical or microscopical signs of colonic inflammation were detected. In both, mice and rats, real or simulated colonic dysbiotic states were also associated to a local modulation of sensory-related markers (endocannabinoid, serotonergic, opioid and vanilloid systems), with specific treatment-related up- and down-regulatory responses (RT-qPCR and immunohistochemistry). These variations at the molecular level translated in functional changes as it relates to visceral pain-related responses. In mice with antibiotic-induced dysbiosis, visceral pain responses assessed using the Writhing test or the intracolonic administration of capsaicin were significantly attenuated when compared to non-dysbiotic animals; thus suggesting a hypoalgesic state. Moreover, colonic contractility assessed in vitro (organ bath) was also altered in dysbiotic mice, indicating a state of increased colonic motility.

Generally, results obtained show that during states of dysbiosis of the GCM there is a complex host response that implies a local immune activation, probably directed towards the reshaping of the microbiota. Data obtained shows that the microbiota is able to influence gut sensory systems and that these changes translate at a functional level in the modulation of visceral pain, eliciting, at least in the present experimental conditions, analgesic-like responses. Similar underlying mechanisms might be responsible for the beneficial effects observed in IBD and, particularly, in IBS patients during antibiotic treatments or during the use of certain bacterial strains as probiotics. Further studies should address the characterization of the specific bacterial groups implicated in these effects. These results highlight the importance of the microbiota as pathogenic factor in gastrointestinal disorders and its potential as a therapeutic approach.

RESUMEN

La microbiota comensal del intestino se considera un factor clave en la homeostasis gastrointestinal. Las alteraciones funcionales gastrointestinales (síndrome del intestino irritable, SII) y las enfermedades inflamatorias intestinales (EII) se han relacionado con alteraciones de la microbiota comensal (disbiosis). Estos pacientes muestran una activación inmune local anormal, con respuestas motoras y sensoriales alteradas, que en el SII se traducen en estados de hipersensibilidad visceral. El papel causal de la microbiota no se conoce con exactitud, pero la presencia de disbiosis y los efectos positivos asociados al tratamiento con antibióticos o ciertos probióticos sugieren un papel destacado.

Este trabajo explora la importancia de la microbiota intestinal modulando los sistemas sensoriales intestinales relacionados con el dolor visceral y sus efectos en respuestas nociceptivas viscerales. Para ello, se ha trabajado con ratas y ratones en los cuales se ha inducido un estado real (cambios adaptativos espontáneos, cambios inducidos por tratamiento con antibióticos) o simulado (estimulación directa de sistemas de interacción hospedador-microbiota) de disbiosis cólica. La microbiota (luminal y adherida al epitelio) se caracterizó usando hibridación in situ con sondas fluorescentes (FISH) y qPCR. La respuesta inmune local y los mecanismos de interacción hospedador-microbiota se valoraron determinando cambios en la expresión génica (RT-qPCR) de citoquinas, péptidos antimicrobianos, integrinas y receptores de tipo Toll (TLR), la producción de IgA secretora, alteraciones histopatológicas y el estado de la barrera de moco. Simultáneamente, se evaluaron cambios en la expresión de marcadores sensoriales. La sensibilidad visceral se valoró mediante el test de *Writhing* o la administración intracólica de capsaicina.

La disbiosis cólica inducida por antibióticos, pero no la observada durante un proceso de adaptación espontánea al ambiente, se asoció a un estado de activación inmune local caracterizado por una regulación selectiva (tanto al alza como a la baja) de citoquinas pro-inflamatorias y de marcadores de interacción hospedador-microbiota y por cambios en los niveles luminales de IgA. Respuestas similares se observaron cuando se simuló al simular un estado de disbiosis mediante la estimulación local del TLR4 (lipopolisacárido) o del TLR7 (imiquimod). En ningún caso se observaron signos macroscópicos o microscópicos de colitis. Tanto en la rata como en el ratón, los estados de disbiosis cólica, real o simulada, se asociaron a una modulación local de la expresión de marcadores sensoriales (sistemas endocanabinoide, serotonérgico, opioide y vaniloide). Se observaron tanto regulaciones al alza como a la baja (RT-qPCR/inmunohistoquímica) dependiendo del modelo de disbiosis y del marcador sensorial considerado. Estos cambios moleculares se tradujeron en cambios funcionales relacionados con respuestas nociceptivas viscerales. Así, en ratones con disbiosis cólica inducida con antibióticos, las repuestas de dolor visceral determinadas con el test de Writhing o tras la administración intracólica de capsaicina mostraron una atenuación significativa con respecto a las observadas en animales control, sugiriendo un estado de hipoalgesia. Estos animales mostraron además una contractilidad cólica alterada (baño de órganos), indicativa de un estado de hipermotilidad.

Estos resultados muestran que en estados de disbiosis intestinal se produce una activación inmune local, dirigida, probablemente, a la restauración de la composición de la microbiota. Se observa que la microbiota es capaz de modular la actividad de los sistemas sensoriales intestinales, generando cambios funcionales que se traducen, en las condiciones experimentales presentes, en una modificación de las respuestas de dolor visceral compatible con un estado de tipo analgésico. Mecanismos similares podrían explicar los efectos beneficiosos asociados al tratamiento con antibióticos o al uso de probióticos observados en pacientes con SII o EII. Estudios posteriores deberían centrarse en la caracterización de los grupos bacterianos específicamente responsables de estos efectos. Estos resultados muestran la importancia de la microbiota como factor patogénico en las alteraciones gastrointestinales y su interés como aproximación terapéutica para las mismas.

RESUM

La microbiota comensal de l'intestí es considera un factor clau en la homeòstasis gastrointestinal. Les alteracions funcionals gastrointestinals (la síndrome de l'intestí irritable, SII) i la malaltia inflamatòria intestinal (MII) s'han relacionat amb alteracions de la microbiota comensal (disbiosi). Els pacients d'aquestes malalties presenten una activació del sistema immune local anormal, amb respostes motores i sensorial alterades, que, en el SII, es tradueixen en un estat d'hipersensibilitat visceral. El paper causal de la microbiota no es coneix amb exactitud, però la presència de disbiosi i els efectes positius associats al tractament amb determinats antibiòtics o probiòtics en suggereixen un paper destacat.

Aquest treball explora la importància de la microbiota intestinal modulant els sistemes sensorials del mateix relacionats amb el dolor visceral i els efectes en respostes nociceptives viscerals. Per aquest motiu, s'ha treballat amb rates i ratolins, en els quals se'ls ha induït un estat real (per canvis adaptatius espontanis, o pel tractament amb antibiòtics) o simulat (estimulació directa de sistemes d'interacció hoste-microbiota) de disbiosi colònica. La microbiota (luminal i adherida a l'epiteli) es va caracteritzar mitjançant hibridació in situ fluorescent (FISH)i qPCR. La resposta immune local i els mecanismes d'interacció hoste-microbiota es van valorar determinant canvis en l'expressió gènica (RT-qPCR) de citocines, pèptids antimicrobians, integrines i receptors de tipo-toll (TLR), la producció d'IgA secretada, avaluant alteracions histopatològiques i l'estat de la barrera del moc intestinal. Simultàniament, es van valorar canvis en l'expressió de marcadors sensorials. Finalment, la sensibilitat visceral es va determinar mitjançant el test de *Writhing* o l'administració intracolònica de capsaïcina.

La disbiosi colònica induïda amb antibiòtics, però no la observada durant processos d'adaptació espontània a l'ambient, es va associar a un estat d'activació immune local caracteritzat per una regulació selectiva (tant a l'alça com a la baixa) de citocines pro-inflamatòries i de marcadors d'interacció hoste-microbiota i, per canvis en els nivells luminals d'IgA. Respostes similars es van detectar al simular un estat de disbiosi mitjançant l'estimulació local del TLR4 (amb lipopolisacàrid) o del TLR7 (amb imiquimod). En cap cas es van observar signes macroscòpics/microscòpics de colitis. Tant en la rata com en el ratolí, els estats de disbiosi colònica, real o simulada, s'associen a una modulació local de l'expressió de marcadors sensorials (principalment dels sistemes endocanabinoide, serotoninèrgic, opioide i vaniloide). Depenent del model de disbiosi i del marcador sensorial considerat, aquesta modulació va implicar canvis, tant a l'alça com a la baixa (RT-qPCR/immunohistoquímica). Els canvis moleculars es van traduir en canvis funcionals relacionats amb respostes nociceptives viscerals. Tanmateix, en ratolins amb disbiosi colònica induïda amb antibiòtics, les respostes de dolor visceral, determinades amb el test de Writhing o amb l'administració intracolònica de capsaïcina, van mostrar una atenuació significativa respecte a les mostrades pels animals control, suggerint un estat d'hipoalgèsia. A més a més, aquests animals van mostrar la contractilitat colònica alterada (amb bany d'òrgans), assenyalant un estat d'hipermotilitat.

Aquests resultats assenyalen que en estats de disbiosi intestinal es produeix un activació immune local, dirigida, probablement, a la restauració de la composició de la microbiota comensal. Sembla que la microbiota es capaç de modular l'activitat dels sistemes sensorials intestinals, generant canvis funcionals que es tradueixen, en les condicions experimentals presents, en una modificació de les respostes de dolor visceral compatible amb un estat analgèsic. Mecanismes similars podrien explicar els efectes beneficiosos associats al tractament amb antibiòtics o a l'ús de probiòtics descrits en pacients amb SII o MII. Els estudis posteriors s'haurien de centrar en la caracterització dels grups bacterians responsables d'aquests efectes. Els resultats presentats evidencien la importància de la microbiota com a factor patogènic o curatiu en les alteracions gastrointestinals i el seu interès com aproximació terapèutica per a les mateixes.

INTRODUCTION

1. THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract is a continuous tubular structure that goes from the mouth to the anus. The intestinal region is composed of the small (duodenum, jejunum and ileum) and the large (cecum, colon and rectum) intestine. Main intestinal functions include transport of the food bolus, enzymatic digestion, absorption of water/electrolytes/nutrients and protection against the external environment (barrier function). As it relates, in particular, to the large intestine, its primary function is to dehydrate and store fecal materials. In this work, we have focused on the large intestine and, particularly, in the ceco-colonic region.

1.1. STRUCTURAL ORGANIZATION

From a structural (histological) point of view, the intestine is formed by four tissue layers (from the inner luminal part to the outside): mucosa (epithelium, lamina propria and muscularis mucosae), submucosa (with blood and lymphatic vessels), muscularis propria (composed of two smooth muscle layers, the inner circular layer and the outer longitudinal layer) and serosa (covering layer of connective tissue) (Figure 1). A particular characteristic of the gastrointestinal tract is the presence of an intrinsic nervous system (enteric nervous system, ENS). Within the intestine, the ENS presents a clear distribution in two neuronal plexuses localized within the submucosa (submucosal plexus) and between the circular and longitudinal smooth muscle layers (myenteric plexus) (Figure 1).

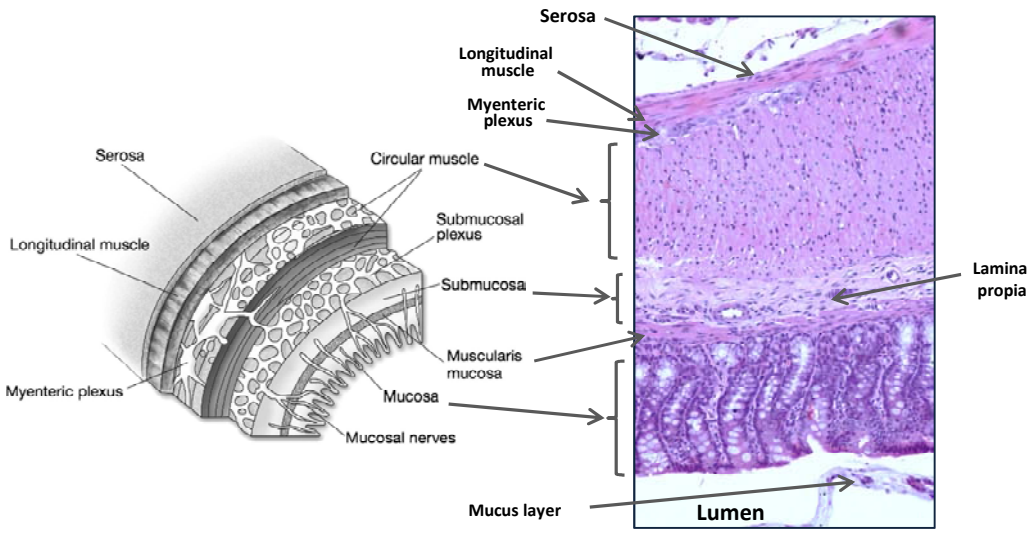


Figure 1. Histological structure of the rat colon.

1.2. Epithelial Cell Types

Histologically, the epithelial lining of the large intestine is organized into multiple crypts associated with a flat luminal surface. The mucosa is composed of tubular glands (Lieberkühn glands) and the crypts are lined by different epithelial cells types (columnar absorptive enterocytes, goblet cells, enteroendocrine cells, Paneth cells and stem cells) (Figure 2). Epithelial regeneration depends on the crypt base, where stem cells divide to produce proliferative progenitors. Cell proliferation finish when differentiated cells exit the crypt, and these epithelial cells continue migrating upwards along the villi and replace the epithelial cells lost via programmed cell death at the villus tip (Barker, 2014).

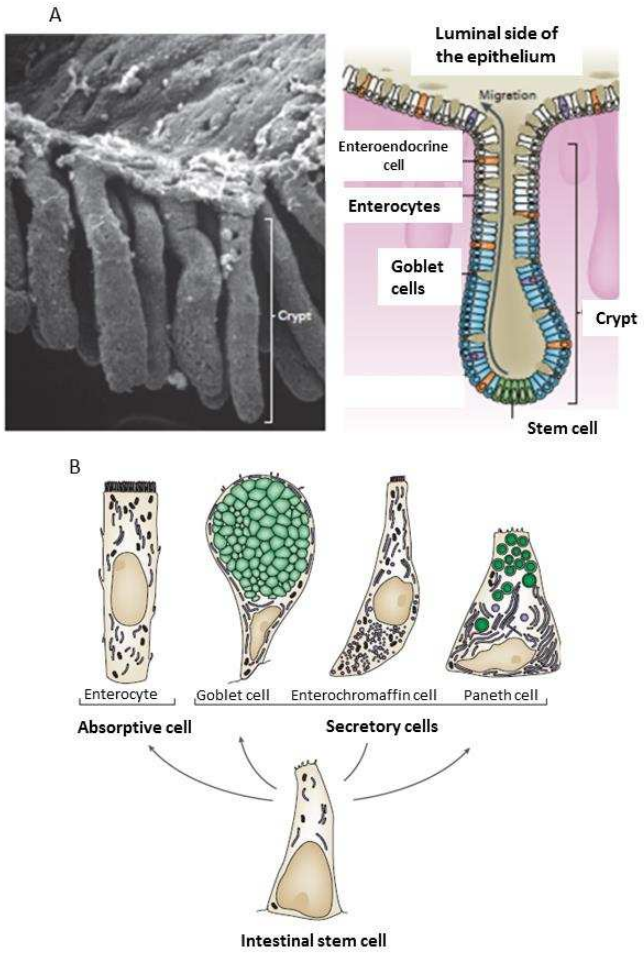


Figure 2. Structural organization of the colonic epithelium. A: Scanning electron micrograph and schematic representation of a colonic crypt. B: Main types of intestinal epithelial cells. Adapted from Barker (2014) and Crosnier, Stamataki, & Lewis (2006).

Enterocytes, specifically colonocytes if they are located in the colon, are absorptive columnar cells with an apical (luminal) surface covered with closely packed microvilli (tiny projections in the brush border) in order to maximize the surface area.

Enteroendocrine cells are part of the endocrine system and can be found throughout the entire intestine. There are at least 15 subtypes which secrete a large variety of regulatory factors, such as serotonin, somatostatin, motilin, cholecystokinin, vasoactive intestinal peptide or enteroglucagon (Moran *et al.*, 2008; Abreu, 2010).

Goblet cells are localized throughout the entire intestine, with cell density increasing caudally. They are responsible for producing the mucous layer, but also secrete a number of bioactive peptides that participate in defensive mechanisms of the gut, particularly against the microbiota (e.g. Resistin-Like Molecule-beta) (Kim and Ho, 2010).

Paneth cells are cells located in the base of the crypts of the small intestine. They synthetize and secrete a variety of antimicrobial compounds important for immunity and host-defense. In physiological conditions they are almost absent in the colon, but they appear in states of mucosal inflammation (Treuting and Dintzis, 2012; Clevers and Bevins, 2013; Mantani *et al.*, 2014).

Moreover, in the colonic mucosa, there is an important resident immune system, the gut-associated lymphoid tissue (GALT). Additionally, abundant immune-related cells can be found among mucosal cells. These include intraepithelial lymphocytes, plasma cells, macrophages, eosinophils, and mast cells.

2. GUT COMMENSAL MICROBIOTA

The microbial community of the GI tract (gut commensal microbiota, GCM) is composed by bacteria, virus, fungi, protozoa and yeasts. Gut colonization starts at birth and, when completed, it harbors about 100 trillion microbial commensals and symbionts belonging approximately to 5.000 distinct species included in the phyla Firmicutes, Bacteriodetes, Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria (Qin *et al.*, 2010; Kamdar *et al.*, 2013; Sommer and Bäckhed, 2013). Intestinal microbiota is not homogenous; *Proteobacteria* spp. (mainly Enterobacteria) and Lactobacillales are dominant in the small

intestine, whereas the large intestine is colonized mainly by Bacteroidetes and Clostridia. Moreover, density of bacterial cells in the gut increases caudally with the maximal counts (10^{11} to 10^{12}cells/g of content in both human and rodents) in the ceco-colonic region (Figure 3) (Swidsinski et al., 2005; Dinoto et al., 2006; Sekirov et al., 2010; Kamada et al., 2013). Intestinal bacteria can be transient or permanent. Transient bacteria are introduced during adult life; they do not permanently colonize the gut and can have positive (probiotics) or negative (pathogens) effects on the host, or be innocuous. On the other hand, permanent bacteria are long-term colonists of the gut, the true commensals, and can have immunostimulatory effects, the authobionts, or detrimental effects, the pathobionts (Ivanov and Honda, 2012).

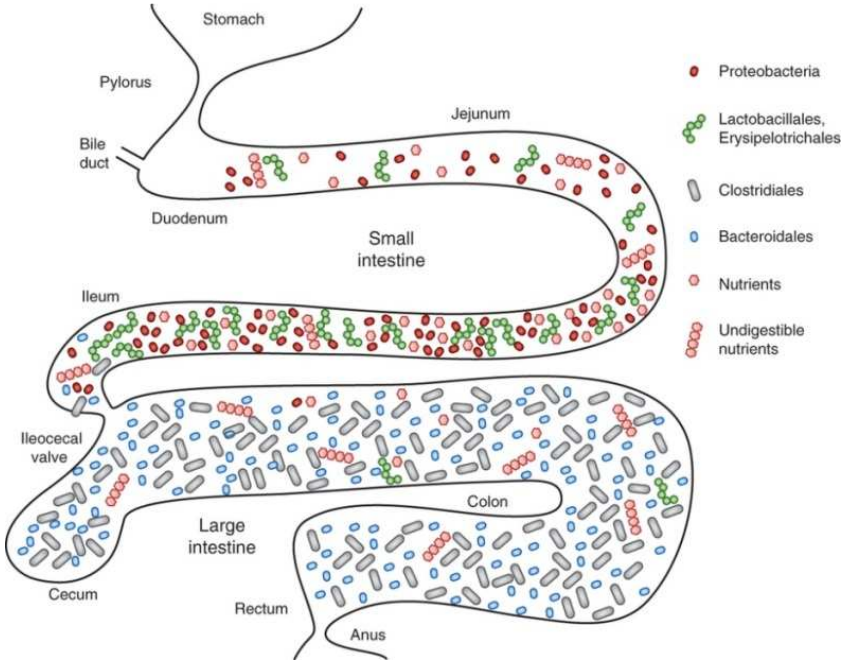


Figure 3. Schematic representation of the gut commensal microbiota: Relative distribution and microbial density along the gastrointestinal tract. From Kamada (2013).

Overall, GCM serves the host giving protection against pathogens, metabolizing complex lipids and polysaccharides and neutralizing drugs and carcinogens; but it can have also potential effects modulating intestinal motility, influencing the maturation of the intestinal immune system and modulating visceral perception (Collins and Bercik, 2009; Montiel-Castro *et al.*, 2013). Changes in the normal composition of the GCM, termed dysbiosis, have been associated with chronic

intestinal diseases such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), as well as with extra-intestinal diseases (e.g. diabetes, multiple sclerosis or psychiatric disorders) (Kamdar *et al.*, 2013; Simrén *et al.*, 2013). Dysbiosis can occur in parallel to intestinal pathogenesis, and can be either a consequence or a cause of the disease (Salzman and Bevins, 2013). In fact, in some intestinal diseases, such as IBS, the causal effects of the microbiota are still a matter of discussion, with some authors considering that dysbiotic states are a consequence and/or a perpetuating factor, rather than a cause, of the disease.

In this work, we have focused on a limited number of bacterial groups, based on their implication in both GI health and disease: *Clostridium* cluster XIVa, Lactobacilli, Bifidobacteria, Bacteroidetes, Enterobacteria and Verrucobacteria. *Clostridium* spp. constitutes one of the largest families of the GCM and, probably due to *C. difficile* infections, has been regarded as a pathogenic group. However, recent data suggests that some members of the clostridia group might have an anti-inflammatory role in immune responses (Barnes and Powrie, 2011; Lopetuso *et al.*, 2013). Moreover, the clostridia-related group of segmented filamentous bacteria (SFB) has been also linked to intestinal inflammation and intestinal immune-regulation (Barnes and Powrie, 2011). On the other hand, Lactobacilli and Bifidobacteria strains are typically considered to confer health benefits to the host, and, because of this, are frequently used as probiotics (Turroni *et al.*, 2014). Specifically, *L. acidophilus* seems to modulate sensory mechanisms leading to visceral analgesia (Rousseaux *et al.*, 2007) while Bifidobacteria can act as immunostimulant (Grangette, 2012). Regarding Gram negative bacteria, Bacteroidetes are the largest group when considering the GCM. Bacteroidetes have been linked to immunomodulatory effects (Round and Mazmanian, 2010), with some specific strains promoting anti-inflammatory responses (Round and Mazmanian, 2010; Barnes and Powrie, 2011). The Enterobacteria group includes several pathogenic strains that cause intestinal infections (such as numerous *E. coli* strains), transfer antibiotic resistance and promote intestinal inflammation (Harmsen et al., 1999; Buffie and Pamer, 2013; Da Re et al., 2013; Terán-Ventura et al., 2014; Yu et al., 2014). Finally, Verrucobacteria are a mucus-degrading group of bacteria that seems to affect intestinal barrier function through the degradation of the epithelial mucus layer (Derrien *et al.*, 2004). Nevertheless, some Verrucobacteria seem to mediate also intestinal immune tolerance (Derrien *et al.*, 2011).

3. IMMUNE HOST-MICROBIAL INTERACTIONS

The intestinal microbiota and food-derived antigens are the main luminal stimuli detected by the host and triggering immune responses within the gut. Some of these stimuli, for instance those generated by the commensal microbiota, should be recognized and tolerated. However, others, such as those generated by pathogenic bacteria, should lead to defensive immune responses. Therefore, within the intestine there is a balance between tolerance and immune responses against microbial-derived stimuli. This balance determines the necessary responses against pathogens while maintaining tolerance to certain antigens and commensals (Geremia *et al.*, 2013).

Both the innate and the adaptive immune systems are involved in these responses, determining an appropriate recognition of the microbiota and the resulting intestinal homeostasis. Therefore, alterations in host-bacterial interactions may result in deregulated intestinal responses to the microbiota, leading to the development of inflammation and even to the generation and/or maintenance of a state of dysbiosis. The innate immune system is highly represented within the GI tract and generates rapid, but fairly unspecific, responses. It includes the physical barrier of mucus layer, dendritic cells, resident macrophages and lymphoid cells and intestinal epithelial cells (Figure 4). (Geremia *et al.*, 2013). On the other hand, the adaptive immune system generates slower, but more specific, responses involving the recruitment of blood-borne immune cells, mostly, but not exclusively, T lymphocytes (Rescigno, 2011; Geremia *et al.*, 2013). Here, we will focus on some of the components of the innate immune system.

3.1. THE MUCUS LAYER

The mucus layer is a dynamic semipermeable barrier which maintains the host separated from the luminal content, including the microbiota (Figure 4). It is divided in two layers: the loosely non-attached outer layer and the inner layer, which is firmly adhered to epithelial cells (Johansson *et al.*, 2011). The mucus layer is formed, mainly, by mucins secreted by goblet cells, together with other secretory products, like trefoil peptides, resistin-like molecule-β and immunoglobulins (Barnett *et al.*, 2012). Goblet cells can be characterized by the type of mucins they contain: acidic, neutral or a mixture of both. Indeed, taking into account their biochemical characteristics, mucins are frequently classified as acidic (they stain blue in a PAS/AB pH=2.5 staining procedure) or

neutral (they satin magenta in a PAS/AB pH=2.5 staining procedure). The mucin composition of the mucus layer varies along the gut. For instance, within the colon, acid mucins are predominant.

Moreover, along the crypts there is also a mucin gradient from the base to the apical part, where the sulphated neutral mucins domain, with the middle part containing a mixture of both acidic and neutral mucins. The composition of the mucus layer can change due to different stimuli; such as stress, diet, bacterial infections or conventionalization of germ free animals. Defects in the mucus layer, likely favoring the interaction with luminal bacteria and their attachment to the epithelium, have been linked to intestinal inflammation (Sharma *et al.*, 1995; Matsuo *et al.*, 1997; Fukushima *et al.*, 1999; Lindén *et al.*, 2008; Johansson, 2014).

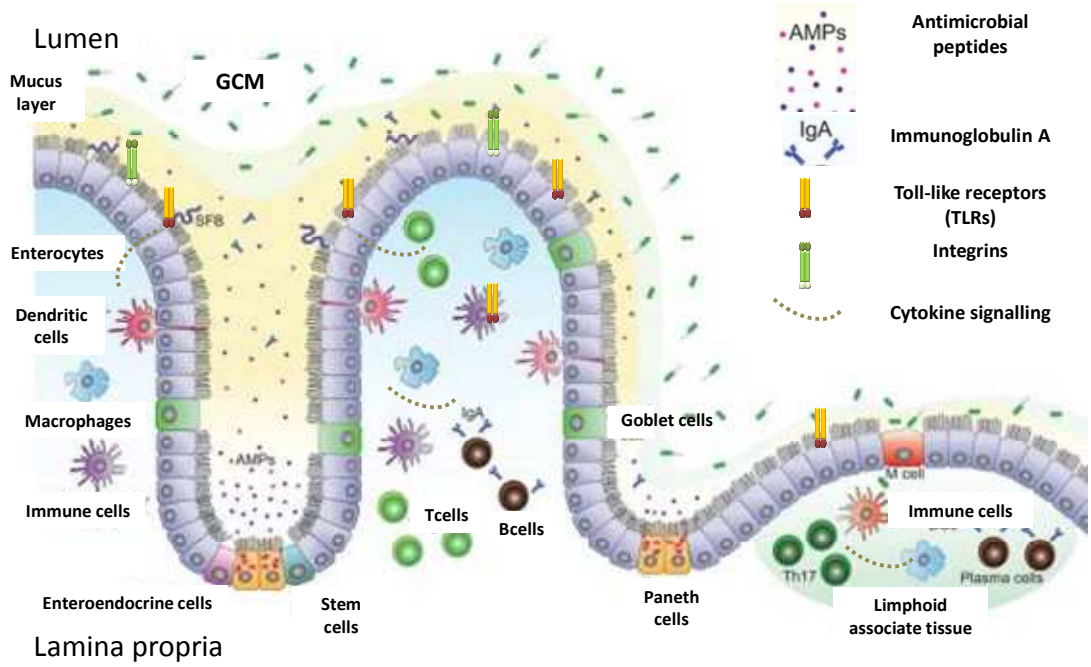


Figure 4. Schematic representation of the main components of the intestinal immune system, including the microbial compartment, the mucus layer, secretory IgA, antimicrobial peptides, cytokines, immune cells and bacterial recognition systems (Toll-like receptors and Integrins). Components of the adaptive immune system are also included. Adapted from Muniz (2012). GCM: Gut Commensal Microbiota; SFB: Segmented Filamentous Bacteria.

3.2. Intestinal Epithelial Cells

Intestinal epithelial cells (IECs) play an important role in innate immune responses by basically: i) forming an essential physical barrier with the intestinal lumen, avoiding the entry of commensal and pathogenic bacteria into the host; ii) sensing the microbial environment by directly interacting with the microbiota that arrives through the mucus layer; and iii) secreting substances with antimicrobial properties, such as antimicrobial peptides (AMPs) (Figure 4). Overall, IECs are involved in many immune-regulatory responses within the gut. Of particular interest is the expression of a series of cell-surface receptors known as integrins. Integrins are involved in cell–cell, cell–extracellular matrix and cell–pathogen interactions (Beaulieu, 1999; Chen *et al.*, 2002). Therefore, they are used by many pathogens for adhesion to the epithelium, and sometimes for cell penetration (Clark *et al.*, 1998; Critchley-Thorne *et al.*, 2006). Integrins are complex molecules composed by different alpha and beta subunits. Within the GI tract the alpha 2 and the beta 1 subunits are wide expressed, being used as markers for these receptors (Beaulieu, 1999; Chen *et al.*, 2002).

3.3. Pattern Recognition Receptors: Toll-like Receptors

Pattern recognition receptors (PRRs) are a series of receptors devoted to sense microorganisms through pathogen-associated molecular patterns (PAMPs) and to detect endogenous stressful signals through danger-associated molecular patterns (DAMPs) (Abreu, 2010). Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NODs) are among the best characterized PRRs. In this work we have focused on TLRs. Within the gut, TLRs are mainly expressed in IECs, although immune cells also express them (Figure 4). TLRs are both cell surface and intracellularly located (Kamdar *et al.*, 2013). To date, in mammals, there are 13 different TLRs subtypes described, with some species-related differences [10 in humans (TLR1–10 and TLR11 as a pseudogene), 12 in mice (TLR1–9 and TLR11–13 and TLR10 as a pseudogenes), and 10 in rat (TLR1-7, 9, 10, 13)] (Table 1) (Albiger *et al.*, 2007; Shibolet and Podolsky, 2007; Cario, 2008; Leulier and Lemaitre, 2008; Vijay-Kumar *et al.*, 2008; Abreu, 2010; Blasius and Beutler, 2010; Gómez-Llorente *et al.*, 2010; Uematsu and Fujimoto, 2010; Brint *et al.*, 2011). TLRs are important for preserving tolerance to commensal microbiota, as well as mediating immune responses against pathogens. TLRs activation induces receptor subtype-specific signaling cascades resulting in the

production of cytokines, chemokines and the transcription of other genes important for controlling infections and inflammatory responses.

In this work we have focused on TLR2, 4, 5 and 7. These TLRs are highly expressed in the GI tract and have been implicated in intestinal neuro-immune responses, both in normal and pathophysiological conditions (Shibolet and Podolsky, 2007; Barajon *et al.*, 2009; Lavelle *et al.*, 2010; McKernan *et al.*, 2011; Sainathan *et al.*, 2012; Kamdar *et al.*, 2013; Saito *et al.*, 2013).

3.4. Secretory-IgA

The intestinal mucosa contains the largest number of antibody secreting plasma cells, which are the main producer of IgA in the GI tract in response to luminal microorganisms or toxins. IgA is exocytosed as a dimeric molecule by IECs into the gut lumen and then is called secretory-IgA (s-IgA) (Figure 4). Within the lumen, s-IgA can recognize, in a rather unselective manner, cell-surface bacterial antigens and covers bacteria, leading to the so called "s-IgA-coated bacteria". S-IgA-coated bacteria seem to be tolerated by the host and have limited interactions with the epithelium. Therefore, s-IgA seems to have a protective role towards the microbiota, as it would prevent the epithelial attachment and translocation of pathogens (Strugnell and Wijburg, 2010; Bemark et al., 2012; Pabst, 2012; Maruya et al., 2013).

In normal conditions, a percentage of the commensal microbiota is covered (coated) by IgA, for this reason it is speculated, and widely accepted, that s-IgA is also controlling GCM (van der Waaij *et al.*, 1996, 2004; De Palma *et al.*, 2010). An increased ratio of s-IgA has been described in states of dysbiosis and/or immune activation of the gut; with variations in the abundance of luminal IgA-coated bacteria (De Palma et al., 2010; Maruya et al., 2013; Martinez-Medina et al., 2014).

Table 1. Mammalian TLRS: Agonists, localization within the gastrointestinal tract and functions.

	Agonists	*Localization (rodent &human)*	*Intestinal function (rodent &human)*
TLR 1	Triacyl lipopeptides (bacteria)	Cell surface: IEC	ND
TLR 2 Heterodimer with TLR 1 and TLR 6	Peptidoglycan Zymosan Triacyl and diacyl lipopeptides(cell-surface lipoproteins) Atypical LPS(bacteria) Phospholipomannan (fungi) Glycosylphosphatidylinositol (protozoa) Envelope protein (virus)	Cell surface: Apical in villi and crypts; Apical and basolateral in lymphoid-associated tissue. Low expression in adult ileum or colon, mainly in crypts.	Chemokine and cytokine production. Protect from apoptosis Involved in AMPs ZO1 redistribution and TFF3 expression Preserved tight junction structure Increased TFF3 expression RELA phosphorylation.
TLR 3	Viral RNA ds DNA Poly(I:C)	Intracellular: Endosomes Neurons and glial cells of the myenteric and submucous plexus. DRG IEC: Basolateral in ileum and colon; top of colonic crypts.	Blocking of TLR3 reduces IL-15 production
TLR 4	Lipopolysaccharide (LPS) Mannan (plants) Glucuronoxylomannan (fungi) Glycoinositolphospholipides (protozoa) Respiratory syncytial virus fusion protein (virus) Endogenous ligands	Cell surface: (Apical in Terminal ileum; basolateral in colon (low); basolateral in ileal crypts; basolateral in colon (low); apical in active Crohn's disease (ileum and colon). Intracellular (in fetal small intestine) Neurons and glial cells of the myenteric and submucous plexus. DRG	Cell growth. Chemokine and cytokine production. Phagocytosis and translocation of bacteria, and uptake of micro particles by M cells; Expression leads to increased TNF production, apoptosis and NF-κB activation; lack of expression leads to decreased TNF production and protects against NEC
TLR 5	Flagellin (bacteria) Gram + and -.	Cell surface: Basolateral in ileum and colon; apical in FAE (small intestine). Intracellular in colon	Chemokine expression Antiapoptotic Pro-inflammatory: NFkB → TNFα
TLR6	Diacyl lipopeptides (bacteria)	ND	ND
TLR 7	RNA ss (viral and no viral); DNA Synthetic imidazoquinolines Guanine analogs (Ioxoribine)	Intracellular: Neurons and glial cells of the myenteric and submucous plexus. DRG	ND
TLR 8	RNA ss (viral and no viral); DNA Synthetic imidazoquinolines	Intracellular: Top of colonic crypts in Ulcerative colitis and Crohn's disease; Not in normal intestine	Chemokine secretion
TLR 9	Unmetilate CpG DNA (bacteria, Protozoa, virus) Hemozoin (protozoa)	Intracellular: Endosomes IEC: apical and basolateral (ileum and colon). In granules and cytoplasm of Paneth cells	Protects against NEC; signaling causes degranulation of Paneth cells
TLR 10	ND	ND	ND
TLR 11	Uropathogenic bacteria Profilin-like molecule (protozoa)	ND - Cell surface	ND
TLR 12	ND	ND	ND
TLR 13	ND	ND – supposed intracellular.	ND

ND: not determined. DRG: Dorsal root ganglia; IEC: intestinal epithelial cell. NEC: necrotizing enterocolitis. From: (Shibolet and Podolsky, 2007; Vijay-Kumar *et al.*, 2008; Barajon *et al.*, 2009; Abreu, 2010; Gómez-Llorente *et al.*, 2010; Uematsu and Fujimoto, 2010; Brint *et al.*, 2011)).

3.5. ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs) are bioactive molecules with a broad-spectrum antimicrobial activity. They are synthetized by a variety of cells, including epithelial cells, Paneth cells, goblet cells and some immune cells. AMPs can be constitutively expressed or secreted in response to the presence of certain microorganisms (Ho *et al.*, 2013; Ostaff *et al.*, 2013). Main AMPs described are:

- *Defensins:* They have activity against Gram-negative and -positive bacteria, fungi, viruses, and protozoa. Defensins are secreted by Paneth cells, epithelial cells, as well as by T cells and neutrophils (Salzman *et al.*, 2007). Main defensins are:

  α-defensins (cryptidins in mice): Constitutively expressed and secreted mainly by Paneth cells and neutrophils. Can promote ion fluxes in epithelial cells or induce secretion of cytokines.

  β-defensins: Constitutively expressed and also induced by endogenous (cytokines) and exogenous (bacterial products) stimuli in response to infection or inflammation. Secreted mainly by IECs.
- *C-type lectins* [regenerating islet-derived protein (REG)]: RegIIIγ is the main REG expressed in the large intestine during pathogen infections or in inflammatory conditions. RegIIIγ is produced in colonocytes and Paneth cells and it is up-regulated in states of TLR activation. It has bactericidal activity against Gram-positive bacteria (Vaishnava *et al.*, 2011; Gallo and Hooper, 2012; Muniz *et al.*, 2012).
- *Resistin-like molecule beta* (RELM β): RELM β is a bioactive molecule (sometimes classified as a cytokine) produced by goblet cells in response to the microbiota. It is involved in intestinal epithelial barrier function (up-regulates mucins' gene expression), preventing bacterial penetration and attenuating intestinal inflammation (Krimi *et al.*, 2008; Muniz *et al.*, 2012).

- *Cathelicidins:* Constitutively expressed and secreted by leukocytes and epithelial cells. They are overexpressed during infection, inflammation and wound healing. Cathelicidins modulate inflammation by altering cytokine responses and by chemo-attraction of inflammatory cells. They might play a secondary role and, therefore, have been much less studied than other AMPs (Chow *et al.*, 2013). Their expression seems to be also TLR-dependent (Chow et al., 2013).

3.6. CYTOKINES

*In t*he intestine, cytokines are synthesized by activated enterocytes and immune cells in the intestinal mucosa, and by immune and enteric glial cells in the muscle layers. Cytokines participate in both innate and adaptive immune responses and can have pro- or anti-inflammatory activity, depending upon de cytokine considered. Main cytokines involved in intestinal inflammation include IL-6, IL-10, IL-12, TNFα and IFNα.

IL-6 (interleukin 6) is a pro-inflammatory cytokine produced by many different cell types, including blood mononuclear cells, intestinal epithelial cells, lamina propria mononuclear cells, fibroblasts and endothelial cells. It is one of the major physiological mediators of the acute phase response and is regarded as a feasible marker of acute inflammatory responses. IL-6 expression is up-regulated in inflammatory conditions of the gut, both in humans and in animal models (Waldner and Neurath, 2014).

IL-12 (which is composed by p35 and p40 subunits) is a pro-inflammatory heterodimeric protein produced by innate immune cells, and induced by bacterial products (Kobayashi *et al.*, 2011). IL-12 mediates in T-cell differentiation and activation, promoting inflammatory responses that, within the GI tract, can lead to the destruction of the intestinal mucosa (Peluso *et al.*, 2006; Atreya and Neurath, 2008).

TNFα (tumor necrosis factor α) is a pro-inflammatory cytokine produced by activated macrophages and lymphocytes. Its major role is the recruitment of circulating inflammatory cells to the target tissue (Atreya and Neurath, 2008). Although TNFα can be up-regulated in several conditions, LPS is considered one of the main inductor of its expression. TNFα seems to be a key immune mediator in intestinal inflammation, as indicated by its high content in the intestinal mucosa in inflammatory bowel disease (IBD) and the efficacy of the anti-TNF therapy in the same patients (Billiet *et al.*, 2014).

IFNα (interferon α type I) is a cytokine produced by macrophages, dendritic cells and IECs and involved in antimicrobial host defense. IFNα expression can be up-regulated by stimulation of TLRs and, in turn, regulates the microbial compartment modulating the activity of IEC and the secretion of AMPs (Katakura *et al.*, 2005; Ludigs *et al.*, 2012; Tschurtschenthaler *et al.*, 2014).

In front of the typical pro-inflammatory role assigned to cytokines, IL-10 (interleukin 10) is a cytokine with demonstrated anti-inflammatory activity. IL-10 is produced mainly by blood

mononuclear cells, macrophages, dendritic cells, epithelial cells and lymphocytes. In the intestine, IL-10 controls the chronic stimulation of the immune system by the commensal microbiota preventing inflammatory responses and maintaining the immune system in balance. IL-10 acts as a compensatory signal against pro-inflammatory stimuli, limiting the secretion of IL-6, IL-12 and TNFα (Maynard and Weaver, 2008; Paul *et al.*, 2012; Shah *et al.*, 2012). Although this key modulatory role, tissue content and expression levels of IL-10 are, in physiological conditions, very low.

4. Intestinal Sensory Systems

Besides the intestinal intrinsic innervations (the ENS), the gut receives also extrinsic nerves from the autonomic nervous system (both sympathetic and parasympathetic). This extrinsic innervation is key to maintain the bidirectional communication with the central nervous system (CNS), and represents the anatomical basis of the gut-brain-gut axis (

Figure 5) (Collins and Bercik, 2009; Ratcliffe, 2011; Collins *et al.*, 2012). Although most of the intestinal functions can be controlled by the ENS, the extrinsic innervation is necessary to maintain a coordinated activity with the rest of the body. In particular, the extrinsic innervation is involved in sensory functions related to visceral pain perception within the gut (

Figure 5). This is particularly important because visceral pain and/or altered visceral sensitivity (hypersensitivity) are frequent symptoms in several gastrointestinal diseases and, in particular, a characteristic feature of irritable bowel syndrome (IBS).

During the last two decades numerous morphological, pharmacological and molecular studies have characterized sensory-related systems within the gut. From these, the serotonergic system, the endocannabinoid system, endogenous opiates and the vanilloid system have received particular attention because of their interest as potential pharmacological target for the treatment of visceral pain.

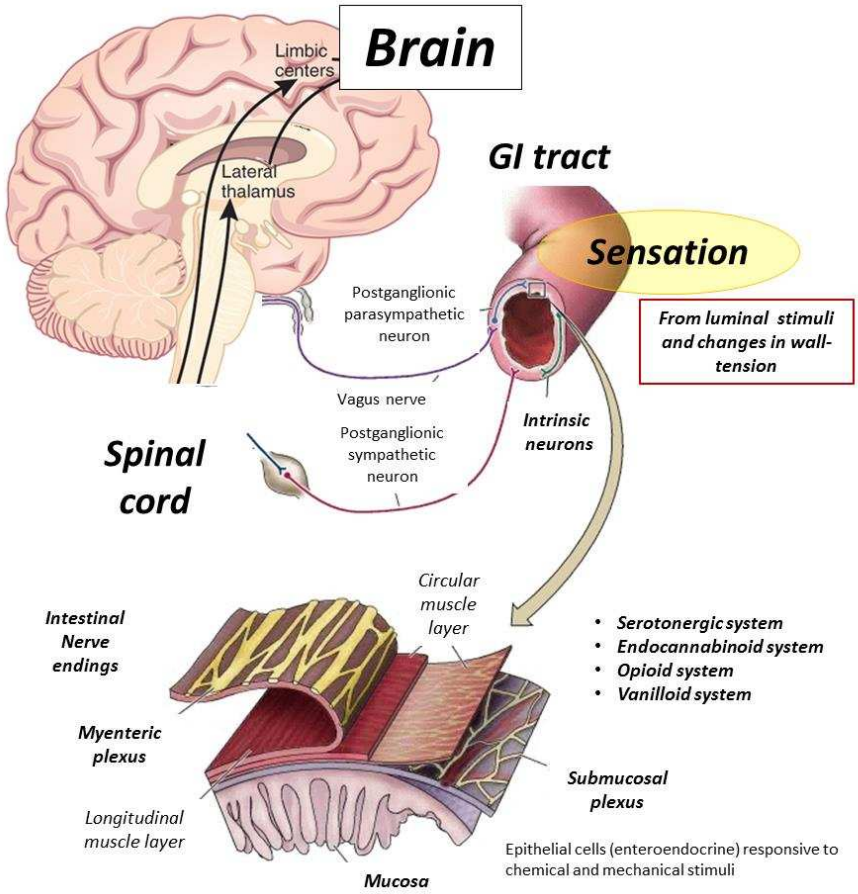


Figure 5. Pathways involved in visceral sensation, from the enteric nervous system (intrinsic innervation) to the extrinsic innervations (postganglionic autonomic, sympathetic and parasympathetic, neurons) and the central nervous system (brain and spinal cord). Sensory signal originate in the intestinal mucosa (such as from luminal chemical stimuli) or from mechanical stimuli affecting the gut wall. Endogenous sensory-related systems (mainly serotonin-, endocannabinoid-, opioid- and vanilloid-mediated) transduce these signals into neural stimuli that, through the afferent extrinsic innervations, arrive to spinal and supraspinal centers. Integration of these signals might lead to the sensation of pain (visceral pain) and the generation of pain-related responses (pain-related behaviors). Adapted from Kuner (2010).

4.1. The Intestinal Serotonergic system

The serotonergic system involves the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) which is stored in the enterochromaffin (EC) cells of the gut mucosa and, to a lesser extent, in neurons of the ENS. Tryptophan hydroxylase (TPH) is the limiting enzyme mediating 5-HT synthesis. There are 2 TPH isoforms: TPH1, mainly expressed in EC cells, and TPH2, expressed in

central and enteric neurons. TPH expression/activity is regarded as a reliable indicator of 5-HT availability, accepting that high expression levels are indicative of a high rate of serotonin production and release (Gershon and Tack, 2007; Ghia et al., 2009; Cremon et al., 2011). Within the GI tract, 5-HT participates in motor, sensory and secretory functions (Camilleri, 2009). 5-HT availability is increased in IBS patients and in animal models of the disease. In addition, some studies have shown that changes in the microbial compartment or stressful conditions can induce 5-HT release from EC cells, leading to the initiation of intestinal inflammation and the generation of abnormal sensory-related responses (altered viscerosensitivity) (Ghia et al., 2009; Kidd et al., 2009; Julio-Pieper et al., 2012; Margolis et al., 2014).

4.2. The Intestinal Opioid system

From a receptor point of view, the endogenous opioid system is composed by three G protein-coupled receptors: μ, δ, and κ opioid receptors. Within the GI tract, intestinal opioids, ligands and receptors, imply myenteric and submucosal neurons and epithelial endocrine and immune cells. As it relates to visceral sensitivity, opioids have a well-characterized analgesic activity (Gray *et al.*, 2006; Holzer, 2009). Their antinociceptive effects are linked to activation of μ and, to a lesser extent, κ receptors. In particular, expression of μ opioid receptors is increased during intestinal inflammation, likely as a compensatory analgesic mechanism generated in states of potentially increased sensitivity. Moreover, recent studies have suggested that probiotics and microbial-related products can module the intestinal expression of μ opioid receptors (Pol *et al.*, 2001; Philippe *et al.*, 2006; Rousseaux *et al.*, 2007; Hutchinson *et al.*, 2009; Sauer *et al.*, 2014).

4.3. The Intestinal endocannabinoid system

The endocannabinoid (CB) system comprises two main receptors, the CB1 and the CB2 (although other putative receptors have been described), together with their endogenous ligands and their metabolizing enzymes (mainly fatty acid amide hydrolase, FAAH). Because of their chemical characteristics, endocannabinoid ligands are difficult to determine; therefore, CB1 and CB2 expression as well as the expression of the FAAH have been used as the main markers to assess the functionality of the endocannabinoid system. Within the GI tract, the endocannabinoid system is involved in controlling intestinal motility, nociception and intestinal inflammation. CB1

and CB2 receptors are expressed on intestinal ganglionic neural cells within the ENS, epithelial cells and immune cells (Wright et al., 2005, 2008; Storr et al., 2008). Specifically, the CB1 receptor predominates in neural and epithelial cells, whereas the CB2 receptors are predominantly expressed in immune cells (Brusberg *et al.*, 2009). Upon activation, both receptors mediate analgesic effects and seem to have also anti-inflammatory activity (Wright *et al.*, 2008; Brusberg *et al.*, 2009; Petrella *et al.*, 2010; De Petrocellis *et al.*, 2012; Zoppi *et al.*, 2012). Probiotics, bacterial products and stressful stimuli have been postulated to influence the endocannabinoid system (Kuiken et al., 2005; Rousseaux et al., 2007; Hong et al., 2009; Alhouayek and Muccioli, 2012).

4.4. THE INTESTINAL VANILLOID SYSTEM

The vanilloid system is one of the six subfamilies of the transient receptor potential (TRP) channel family. There are 6 types of Transient Receptor Potential Vanilloids (TRPV1-6) (Nilius and Mahieu, 2006). These receptors are calcium permeable, non-selective cation channels involved in thermo- and chemo-sensitive transduction (Venkatachalam and Montell, 2007). In the intestine, TRPV1, 3 and 4 have been linked to viscerosensitivity as pro-algesic receptors (Venkatachalam and Montell, 2007; Phillis *et al.*, 2009; Izzo and Sharkey, 2010; De Petrocellis *et al.*, 2012). In the GI tract, TRPV are expressed mainly in afferent nerves, although they can also be found in enteroendocrine epithelial and immune cells (Ueda *et al.*, 2009; Blackshaw, 2014a, 2014b). In agreement with their pro-algesic effects, TRPV are up-regulated in states of intestinal inflammation and visceral hypersensitivity (Holzer, 2008; Boesmans *et al.*, 2011; Vergnolle, 2014).

4.5. ASSESSMENT OF VISCERAL SENSITIVITY IN ANIMAL MODELS

During the last 20 years many efforts in the study of visceral pain have been done to obtain reliable animal models. Overall, two groups of models have been used in the literature, depending upon the noxious stimuli used to elicit pain; those based on the mechanical stimulation of the gut and those based on the chemical stimulation. None of these models is likely to reflect the whole spectrum of mechanisms/processes involved in visceral pain. Nevertheless, they are accepted tools to assess visceral pain and have shown consistent and reproducible responses to pharmacological treatments (Bulmer and Grundy, 2011; Holschneider et al., 2011).

The colorectal distension (CRD) model is based in the mechanical stimulation of the colorectal area, eliciting the stimulation of mechanosensitive sensory afferents and the generation of pain and the associated viscerosomatic responses (Jones and Gebhart, 2004). On the other hand, the classical Writhing test and the intracolonic administration of capsaicin are the main models based on chemical stimulation. The Writhing test (intraperitoneal administration of diluted acetic acid and observation of the pain-related behaviors elicited) is sometimes regarded as a viscerosomatic pain test, since somatic structures (like abdominal muscles or the peritoneum) can also be irritated and elicit pain (Martínez *et al.*, 1999). The intracolonic administration of capsaicin (a vanilloid substance acting as a TRPV1 agonist) is based on the local stimulation of TRPV1 (Laird *et al.*, 2001). Because of the redundancy of the system and the interplays described between the vanilloid and the endocannabinoid and opioid systems (De Petrocellis and Di Marzo, 2009), capsaicin is likely to generate a cascade that leads to the generation of a sensory-related response that implies multiple sensory components and not exclusively the TRPV system. Therefore, local capsaicin will elicit an afferent stimulation that, when integrated within the CNS, will result in a series of quantifiable pain-related responses (Laird *et al.*, 2001).

5. COLONIC MOTILITY AND MICROBIOTA

Colonic motility refers to spontaneous movement which in the colon results in mixing/turnover, propulsion, or both, of the luminal contents from oral to aboral direction. There are three distinct types of contractions: 1) rhythmic-phasic contractions, 2) propulsive contractions, and 3) tonic contractions. Motility patterns are generated by a complex interaction of enteric motor neurons, smooth muscle cells and interstitial cells of Cajal and can be influenced by numerous factors, including stress or inflammation (Camilleri and Ford, 1998; Spencer, 2001; Sanders, 2008; Sarna, 2010; Reigstad and Kashyap, 2013).

A detailed description of the motility of the colon and its mechanisms of control is out of the scope of the present work. Nevertheless, it is important to notice that colonic dysmotility is frequently observed in states of dysbiosis (such as during enteric infections), and is, together with altered viscerosensitivity, a key finding in IBS patients. Therefore, together with viscerosensitivity,

motility might be one of the aspects to consider when assessing the role of microbiota within the GI tract.

6. Functional and Inflammatory Gastrointestinal Disorders and Gut Microbiota

Besides cancer, the main alterations of the gastrointestinal tract are functional gastrointestinal disorders (FGIDs, mainly IBS) and inflammatory conditions (Inflammatory Bowel Disease, IBD). As mentioned previous, the gut microbiota seem to be an important pathogenic component of these conditions.

IBS has a multifactorial etiology and pathophysiology, which is attributed to alterations in gastrointestinal motility, and visceral sensitivity, with a dysfunction of the gut-brain-gut axis and the implication of certain psychosocial factors (such as stress) (Longstreth *et al.*, 2006; Ringel *et al.*, 2009; Saulnier *et al.*, 2013). IBS is not associated to overt structural alterations (inflammation). However, nowadays it is widely accepted that the disease is associated with a low-grade inflammation (without histopathological evidence) and/or an abnormal local immune function (Ringel *et al.*, 2009; Akiho *et al.*, 2010; Ohman and Simrén, 2010; Simrén *et al.*, 2013).

Stress is one of the main environmental causes linked to IBS, either initiating or exacerbating the pathology (Spiller *et al.*, 2007; Vicario *et al.*, 2012). Acting via de gut-brain-gut axis stress alters motility, barrier function and visceral sensitivity. A crucial role for the microbiota has been suggested in these effects since stressful conditions can break the epithelial barrier allowing the entrance of luminal antigens (including microbial-derived product and bacterial translocation) and facilitating altered (enhanced) immune responses to the same luminal factors (Bailey *et al.*, 2006, 2011; Larauche *et al.*, 2011, 2012; Dinan and Cryan, 2012). Through these effects stress is likely to influence microbiota, although this action has not been demonstrated (Bailey et al., 2011; Larauche et al., 2011; de Jonge, 2013; Fukudo, 2013; Montiel-Castro et al., 2013).

IBD is an inflammatory condition in which genetically susceptible individuals with a deregulated immune response can, under certain environmental conditions, develop bowel inflammation. Common symptomatology includes abdominal pain and extra-intestinal

manifestations (Melgar and Shanahan, 2010; Baumgart and Sandborn, 2012; Ordás *et al.*, 2012). Moreover, patients with IBD in remission can also suffer from IBS-like symptoms (Ohman and Simrén, 2010).

Nowadays it is widely accepted that GCM plays a role in the initiation, development/maintenance and resolution of both functional and inflammatory intestinal conditions. However, the causal effects are not clear and microbial changes might be regarded as either a cause or a consequence of the disease state. Nevertheless a role for GCM is supported by: i) the evidence that IBD and IBS patients present intestinal dysbiosis; ii) the beneficial effects observed with some treatments with antibiotics; and iii) the beneficial effects observed upon treatment with certain bacterial strains used as probiotics (Gionchetti et al., 2006; Quigley, 2011; Dai et al., 2013; Hungin et al., 2013; Chang, 2014).

HYPOTHESIS AND OBJECTIVES

From the background presented we can conclude that, besides the classical neuro-immune-endocrine regulatory mechanisms, the gut commensal microbiota (GCM) should be regarded as an active component in gastrointestinal homeostasis, with numerous evidences suggesting the existence of a host-bacterial axis that influences intestinal and extra-intestinal functions. Consequently, alterations in GCM and its interaction with the host can lead to pathophysiological states, likely including intestinal inflammation and altered secretomotor and sensory functions.

Therefore, we **HYPOTHESIZED** that imbalances in the GCM might have a potential influence in host-bacterial interactions systems and might affect intestinal sensory-related mechanisms, leading, as a final consequence, to functional alterations in visceral sensitivity.

To proof this hypothesis we characterized, at molecular and cellular levels, immune- and visceral sensory-related changes in different states of colonic dysbiosis (spontaneous adaptive changes or antibiotic-induced dysbiosis) or when dysbiosis is simulated by direct stimulation of TLR-dependent host-bacterial interaction systems in rodents (rats or mice). Furthermore, we also assessed if these changes might have functional consequences in visceral sensitivity.

Taking this into account, the specific **OBJECTIVES** of this work were:

- To determine if spontaneous changes of the GCM related to the adaptation to new environmental conditions are associated to changes in sensory-related systems within the colon.
- To characterize local changes in host-bacterial interaction systems and sensory-related systems in states of colonic dysbiosis.
- To determine if potential neuro-immune changes associated to colonic dysbiosis translate into functional changes related to visceral sensitivity or colonic motility.
- To characterize the interaction between psychological stress and GCM and their potential role modulating functional colonic responses to stress.
- To assess if dysbiotic states of the colon are associated to the induction of colonic inflammation.

CHAPTER 1

ENVIRONMENT-RELATED ADAPTIVE CHANGES OF GUT COMMENSAL MICROBIOTA DO NOT ALTER COLONIC TOLL-LIKE RECEPTORS BUT MODULATE THE LOCAL EXPRESSION OF SENSORY-RELATED SYSTEMS IN RATS

M. Aguilera[a,c], P. Vergara[a,b,c], V. Martínez[a,b,c]

[a]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain.

[b]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Spain.

[c]Neuroscience Institute, Universitat Autònoma de Barcelona, Barcelona, Spain.

ABSTRACT

Pathogenic and protective roles have been attributed to gut commensal microbiota (GCM) in gastrointestinal inflammatory and functional disorders. We have shown that the adaptation to a new environment implies specific changes in the composition of GCM. Here we assessed if environment-related adaptive changes of GCM modulate the expression of colonic Toll-like receptors (TLRs) and sensory-related systems in rats. Adult male SD rats were maintained under different environmental conditions: barrier-breed-and-maintained; barrier-breed adapted to conventional conditions or conventional-breed-and-maintained. Fluorescent *in situ* hybridization and real-time quantitative PCR (qPCR) were used to characterize luminal ceco-colonic microbiota. Colonic expression of TLR-2, -4, -5 and -7, cannabinoid receptors (CB1/CB2), μ-opioid receptor (MOR), transient receptor potential vanilloid (TRPV1, 3 and 4), protease-activated receptor 2 (PAR-2) and calcitonin-gene related peptide (CGRP) were quantified by qPCR. CB1/CB2 and MOR expression were evaluated also by immunohistochemistry. In rats, housing-related environmental conditions induce specific changes of GCM, without impact on the expression of TLRs-dependent bacterial recognition systems. Expression of sensory-related markers (MOR, TRPV3, PAR-2 and CB2) decreased with the adaptation to a conventional environment; correlating with changes in *Bacteroides* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. counts. This suggests an interaction between GCM and visceral sensory mechanisms, which might be part of the mechanisms underlying the beneficial effects of some bacterial groups on functional and inflammatory gastrointestinal disorders.

Keywords: Cannabinoid receptors, Gut commensal microbiota, Mu opioid receptor, Protease-activated receptor 2, Toll-like receptors, Vanilloid receptors

Chapter 1

INTRODUCTION

Gut commensal microbiota (GCM) is a dynamic microbiological system comprised by a large number of bacterial species.[1] Numerous evidences support a critical role for commensal bacteria in the maintenance of gut homeostasis. For instance, GCM appears to be of crucial importance in the modulation of local immune responses. [2] and, through these mechanisms, to be a pathogenic component of inflammatory and functional gastrointestinal alterations, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Indeed, several reports suggest that GCM composition is altered in both IBD and IBS patients. [3–5] Therefore, modulation of the commensal microbiota, mainly through the administration of probiotics and/or prebiotics, has become an attractive, and largely explored, approach for the treatment of these alterations. This is based in numerous studies, both in animals and humans, suggesting that specific microbial agents might modulate immune responses, local epithelial functions, motor activity, afferent sensory information associated to visceral sensitivity and even central nervous system responses associated to intestinal stimuli. [3,6–9] However, the mechanisms mediating these effects remain largely unknown.

Recent data derived from animal models of IBS evaluating sensory information arising from the gut suggest that GCM might influence neuronal and/or neuro-immune mechanisms within the gut.[3] In mice and rats, modifications of the intestinal commensal microbiota result in changes in nociceptive responses that have been associated to alterations in neuro-immune mediators implicated in sensory mechanisms, including pain responses, within the gut.[10,11] We have recently shown that GCM has spontaneous, environmental-related, adaptive changes. In particular, animals breed under barrier conditions, when moved to standard housing conditions present an adaptive shift in their GCM towards the characteristic or the new environment.[12] Similar environmental-related changes have been also described in mice.[13,14] The significance of these changes is not clear but might implicate alterations in gut homeostasis leading to altered functional responses and/or the development of pathophysiological states.

Based on these observations, we aimed to correlate environmental-related adaptive changes in GCM with changes in neuro-immune sensory systems within the gut. For this, we characterized spontaneous changes of ceco-colonic commensal microbiota, using fluorescence *in situ* hybridization (FISH), in rats born and bred under different environmental conditions. FISH results were confirmed by real-time quantitative PCR (qPCR). In the same animals the gene

expression of cannabinoid receptors (CB1 and CB2), transient receptor potential vanilloid channels (TRPV1, TRPV3 and TRPV4), protease-activated receptor 2 (PAR-2), μ-opioid receptors (MOR) and calcitonin-gene related peptide (CGRP) was assessed also by qPCR. Moreover, the intestinal expression of cannabinoid receptors (CB1 and CB2) and MOR was also evaluated by immunohistochemistry. All these receptors/mediators participate in sensory and secretomotor responses within the gut and have been implicated in both inflammatory and functional gastrointestinal alterations.[15–19]

The interaction between gut microbiota and the host is known to be mediated through pattern recognition receptors (PRR). Toll-like receptors (TLRs) are among the best characterized PRRs; they are largely expressed within the gut and are key components mediating bacterial-host interactions and microbial recognition.[20–23] Therefore, we also assessed if environmental-related changes in the luminal GCM affect the local expression of TLRs, specifically the TLR subtypes 2, 4, 5 and 7 (TLR2, TLR4, TLR5 and TLR7).

MATERIALS AND METHODS

ANIMALS

Four 6 week old and four 9 week old male OFA Sprague-Dawley rats (SPF, specific pathogen free) bred and maintained in a barrier protected area with all materials, water, food and bedding sterilized before entering the barrier were obtained from Charles River Laboratories (Lyon, France). The original microbiota inoculated in these animals (as provided by the breeder) consisted of: *Bacteroides distasonis*, *Lactobacillus acidophilus*, *Lactobacillus salivaris*, Schaedler fusiform-shaped bacterium, 3 strains of CRL fusiform-shaped bacterium,CRL mouse spirochete, *Escherichia coli* (non haemolytic), *Streptococcus faecalis* (group D) and *Enterococcus* spp. In addition, four 9 week old male OFA Sprague-Dawley rats bred in conventional conditions in the Animal Facility of the Universitat Autònoma de Barcelona were used. This conventional colony was established in 1994 from OFA Sprague Dawley rats from Charles River Laboratories (Lyon, France) and has been appropriately cross-bred in order to maintain genetic stability. When in conventional conditions, water, food and bedding were given to animals as facilitated by the commercial provider, without any further treatment.

All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya.

EXPERIMENTAL GROUPS AND SAMPLE COLLECTION

Three experimental groups were defined: 1) 9 week old barrier-bred rats (barrier-breed-and-maintained group; n=4); 2) 9 week old rats born and bred under conventional conditions (conventional-breed-and-maintained group; n=4); and 3) 6 week old barrier rats maintained under conventional conditions at the animal facility of the UAB for 3 weeks (barrier-breed adapted to conventional conditions group; n=4). All animals were 9 week old at the time of testing. To avoid potential time-related changes in microbiota all experimental groups were processed simultaneously.

Animals were euthanized by CO_2 inhalation followed by a thoracotomy. Thereafter, the abdominal cavity was opened, the ceco-colonic region localized and fecal content and tissue samples were collected and frozen immediately with liquid nitrogen. All samples were stored at -80 ºC until analysis. For immunohistochemistry analysis a 3 cm segment of the mid-portion of the colon was removed and fixed overnight with 4% paraformaldehyde.

ENUMERATION OF BACTERIA USING FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

For FISH, general methods previously reported were followed.[12,24] Oligonucleotide probes consisted of a single strain DNA covalently linked with Cy3 at the 5'-end (see Table 1 for details of the probes used). All probes were obtained from Tib MolBiol or Biomers.

Frozen fecal contents (about 0.5 g) were thawed and 4.5 ml of Millipore filtered PBS 1x, at pH 7.2, and 3-5 glass beads (3mm diameter) were added and homogenized on a vortex for 3 min. The suspension obtained was then centrifuged for 1 min at 700 g. 1 ml of the supernatant was collected and fixed overnight (4 ºC) in 3 ml of freshly prepared 4% paraformaldehyde and stored at –20 ºC until analyzed.

At the time of analysis, samples were diluted in PBS 1x and spotted on pre-cleaned, gelatin-coated [0.1% gelatin, 0.01% $KCr(SO_4)_2$] Teflon printed slides (10 wells, diameter 8mm; K-11, Knittel Gläss), dried at room temperature and dehydrated in 96% ethanol (10 min). Samples were hybridized in a dark moist chamber by addition of 100 µl hybridization buffer (20mM Tris-HCl, 09M

NaCl, 0.1% SDS at pH 7.2) preheated with the corresponding Cy3-labeled oligonucleotide probe (with a final concentration of 10ng/µl) in each well. Hybridization temperatures and duration were used as previously described to achieve the optimal stringency.[12] Lysozyme treatment (37ºC, 90 min) was performed prior to the hybridization process for detection of *Lactobacillus* spp.

After hybridization, the slides were rinsed in a pre-warmed washing buffer (20mM Tris-HCl, 0.9M NaCl at pH 7.2) for 30 min, at each hybridization temperature, and then cleaned with Milli-Q water to remove unbound probes. Washed slides were air-dried and mounted with Vectashield® Mounting Media with-DAPI (H-1200; Vector Laboratories) to stain the chromosomes as a control signal.

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; Carl Zeiss Microscopy). For quantification of bacteria, 20 randomly selected fields were photographed and the number of hybridized cells counted using the CellC software.[25]

Relative quantification of bacteria using real-time quantitative PCR (qPCR)

Total DNA was isolated from frozen ceco-colonic content using QIAamp® DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. Thereafter, DNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), diluted to equal concentrations with sterile deionised water and stored at -20ºC until analysis.

The relative abundance of bacteria was measured using 16S rRNA gene-targeting hydrolysis probes (Custom TaqMan assays; Applied Biosystems; see Table 1 for details of the different probes used). Amplifications were carried out in a final volume of 20µL [1µL of assay (Primers: 18µM and Probe: 5µM), 10 µL of TaqMan Universal Master Mix II, 5 µL of Milli-Q water and 4 µL of DNA (40ng for *Bifidobacterium* spp., *Clostridium* spp. *and Bacteroides* spp. and 4 ng for *Lactobacillus* spp.)]. Amplification program was 95ºC during 10 min followed by 40 cycles at 95ºC for 30 s and 60ºC for 1 min. For *Lactobacillus* spp the amplification program was increased to 50 cycles. All samples and the negative controls were assayed for triplicate. The barrier-breed-and-maintained group served as the calibrator.

Table 2. Primers (FISH) and probes (qPCR) used for quantification of bacterial 16S rRNA genes.

	FISH			qPCR	
	Probe	**Cy3 - 5' – 3'**	**Ref.**	**FAM - 5' – 3'**	**Ref.**
Non bacteria	NON 338	ACATCCTACGGGAGGC	12,24		
Total bacteria	EUB 338	GCTGCCTCCCGTAGGAGT	12,24		
Enterobacteria	ENT-D	TGCTCTCGCGAGGTCGCTTCTCTT	12,24		
***Bacteroides* spp**	BAC 303	CAATGTGGGGGACCTT	12,24	AGAGGAAGGTCCCC GCTACTTGGCTGGTT CATTGACCAATATTCCTCACTGCTGC	38
***Bifidobacterium* spp**	BIF 164	CATCCGGCATTACCACCC	12,24	CGTGCTTAACACATGCAA CACCCGTTTCCAGGAG TCACGCATTACTCACCCGTTCG	38
***Clostridium* cluster XIVa**	EREC 482	GCTTCTTAGTCAGGTACCG	12,24	AGTGGGGAATATT TTGAGTTTCATTCTTGC AATGACGGTACCTGA	38
***Lactobacillus* spp**	LAB 158	GGTATTAGCACCTGTTTCCA	12,24	TGGATGCCTTGGCACTAGGA AAATCTCCGGATCAAAGCTTACTTAT TATTAGTTCCGTCCTTCATC	39

F: forward primer; R: revers primer; R: probe.

To determine the relative bacterial content an expression value of 1 was assigned to the barrier-breed-and-maintained group and expression levels in the other groups were referred to it. In these conditions, relative expression values above 1 imply an increase in bacterial counts in relation to the barrier-breed-and-maintained group. Conversely, relative expression values below 1 imply a decrease in bacterial counts in relation to the barrier-breed-and-maintained group.

GENE EXPRESSION USING QUANTITATIVE REVERSE TRANSCRIPTION-PCR (RT-QPCR)

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit, (AM1924; Ambion/Applied Biosystems); thereafter, a Two-step RT-PCR was performed. RNA samples were converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems). Only a consistent 260/280 ratio (between 1.8-2) found with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was accepted to perform a quantitative *real time* PCR using hydrolysis probes. TaqMan gene expression assays for CB1 receptors (Rn00562880_m1), CB2 receptors (Rn00571953_m1), MOR (Rn01430371_m1), TRPV1 (Rn00583117_m1), TRPV3 (Rn01460303_m1), TRPV4 (Rn00576745_m1), PAR-2 (Rn00588089_m1), CGRP (Rn01511354_m1), TLR2 (Rn02133647_s1), TLR4 (Rn00569848_m1),

TLR5 (Rn01411671_s1) and TLR7 (Rn01771083_s1) were used (Applied Biosystems). β-actin (Rn00667869_m1) was used as endogenous housekeeping and the barrier-breed-and-maintained group served as the calibrator.

The PCR reaction mixture was transferred to a MicroAmp optical 96-well reaction plate and incubated on the ABI 7900 HT Sequence Detection System (Applied Biosystems). RQ Manager 1.2 software was used to obtain the cycle threshold for each sample; thereafter all data were analyzed with the comparative Ct method ($2-\Delta\Delta Ct$).[26]All samples and the negative controls were assayed for triplicate.

IMMUNOHISTOCHEMISTRY

Paraffin embedded tissue sections (5 µm thick) were deparaffinized and rehydrated with a battery gradient of alcohols. Antigen retrieval for CB1 receptor and MOR was achieved by processing the slides in a microwave with 10mM of citrate solution. Epitope retrieval for CB2 receptor was performed using a pressure cooker (at full pressure, for 3 min) in Tris-EDTA solution buffer. Quenching of endogenous peroxidase was performed by 1-h incubation with 5% H_2O_2 in distilled water. Primary antibodies included a rabbit polyclonal anti-CB1 (1:100; Rabbit polyclonal to Cannabinoid Receptor 1, ab23703; Abcam), a rabbit polyclonal anti-CB2 (1:100; Rabbit polyclonal to Cannabinoid Receptor 2 (H-60), sc-25494; Santa Cruz Biotechnology) and a rabbit polyclonal anti-MOR (1:2500; Rabbit polyclonal to mu opioid receptor AB1580; Chemicon/Millipore). The secondary antibody used was a biotinylatedpolyclonal swine anti-rabbit IgG (E 0353; DakoCytomation). Detection was performed with avidin/peroxidase kit (Vectastain Elite ABC kit, PK-6100; Vector Laboratories), antigen-antibody complexes were reveled with 3-3'-diaminobenzidine (SK-4100 DAB; Vector Laboratories), with the same time exposure per antibody, and sections were counterstained with haematoxylin. Specificity of the staining was confirmed by omission of the primary antibody.

Quantification of immune-positive signal in the myenteric plexus

For CB2, immunopositive cells were counted in 30, randomly selected, myenteric ganglia, in duplicate, for each tissue sample. Cells were considered to be immunopositive if they expressed more labeling that the background levels seen in the negative controls.

MOR immunoreactive myenteric ganglia were quantified by means of gray density using ImageJ software (NIH Image, USA). A minimum of 15 myenteric ganglia per sample were evaluated. Images were taken with the same light intensity and control negative signals were used to determine the positive grey threshold.

All counting was performed on coded slides to avoid any bias.

Statistical Analysis

Data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for qPCR data. Comparison between multiple groups were performed using a one-way analysis of variance (one-way ANOVA), followed when necessary, by a Student-Newman-Keuls multiple comparisons test. Correlation between parameters was assessed by linear regression and Pearson's analysis. In all cases, results were considered statistically significant when $P<0.05$. All statistical analysis and graphs were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, USA).

Results

Characterization of intestinal (ceco-colonic) commensal microbiota by FISH

Total mean bacterial counts, as determined using DAPI staining and EUB 338 hybridization, oscillated between 5.3×10^9 and 2.2×10^{10} cells/ml, and were comparable in all experimental groups, and within the margins previously described [13, 31]. However, FISH analysis revealed significant differences among groups in the commensal microbiota composition (Table 2; Figs. 1 and 2).

Among Gram negative bacteria, the counts of Enterobacteriaceae (ENT-D probe) were scarce (in many cases less than 1% of the flora quantified), regardless the group considered. In contrast, *Bacteroides* spp. group (BAC 303 probe) was relatively abundant and counts were significantly higher in the barrier-breed-and-maintained and the conventional-breed-and-maintained groups when compared with the barrier-breed adapted to conventional conditions group (Table 2, Figs. 1 and 2).

Table 3. Bacterial counts in the different experimental groups, as determined by FISH ($\times 10^8$ cells/ml).[a]

	Barrier-breed-and-maintained	Barrier-breed adapted to conventional conditions	Conventional-breed-and-maintained
Total cells[b]	171.1±21.5	113.1±34.9	138.0±37.4
Enterobacteria	0.7±0.09	0.7±0.3	1.28±0.2
***Bacteroides* spp**	6.4±2.2	0.428±0.84[&]	2.77±0.7
***Bifidobacterium* spp**	0.2±0.06	0.27±0.17	1.8±0.6
***Clostridium* cluster XIVa**	35.7±11.2	24.8±5.4	45.3±20
***Lactobacillus* spp**	0.3±0.1**	1.28±0.38*	3.4±0.8

[a]: Data are mean±SEM, n=4 per group. Because of technical problems one animal of the Barrier-breed-and-maintained group was not analyzed for *Bifidobacterium* spp.[b]: Total fecal microbial counts as determined by DAPI staining. *, **: P <0.05 or 0.01 vs. Conventional-breed-and-maintained group, &: P < 0.05 vs. Barrier-breed-and-maintained group (ANOVA).

Overall, Gram positive bacteria represented more than 80% of the total FISH-quantified microbiota. In particular, *Clostridium coccoides* - *Eubacterium rectale* group (*Clostridium* cluster XIVa, EREC 482 probe) accounted for the largest bacterial population in all experimental groups. Counts for *Lactobacillus* spp. and *Enterococcus* spp. (LAB 158 probe) and *Bifidobacterium* spp. (BIF 164 probe) were higher in fecal samples from the conventional-breed-and-maintained group when compared with the barrier-breed-and-maintained and barrier-breed adapted to conventional conditions groups, which showed clear transitional changes towards to the adaptation to conventional conditions (Table 2, Figs. 1 and 2).

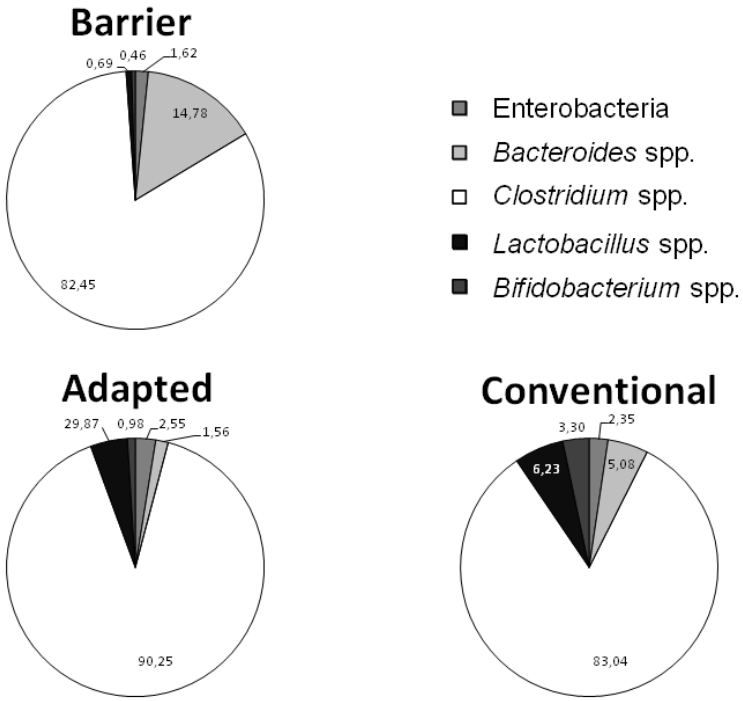


Figure 1. Relative composition of the ceco-colonic microbiota, as quantified by FISH, in the different experimental groups. Data represent relative abundance (%) of the different bacterial groups characterized by FISH (*Bacteroides* spp, Enterobacteriaceae, *Bifidobacterium* spp., *Lactobacillus* - *Enterococcus* spp. and *Clostridium* cluster XIVa group). Relative percent composition was calculated taking as 100% the total counts of the bacterial groups assessed. Barrier: barrier-breed-and-maintained group; Adapted: barrier-breed adapted to conventional conditions group; Conventional: conventional-breed-and-maintained group. See Table 1 for exact cell counts.

CHARACTERIZATION OF INTESTINAL (CECO-COLONIC) COMMENSAL MICROBIOTA BY QPCR

qPCR was used to confirm FISH results. The bacterial groups assessed (*Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp. and *Clostridium* cluster XIVa) were detected in all fecal samples. However, relative expression levels (indicative of the bacterial density) were different among bacterial and among experimental groups. Overall, relative abundance for the different bacterial groups followed the same pattern obtained using FISH (Fig. 2), revealing the same changes associated to the environmental conditions.

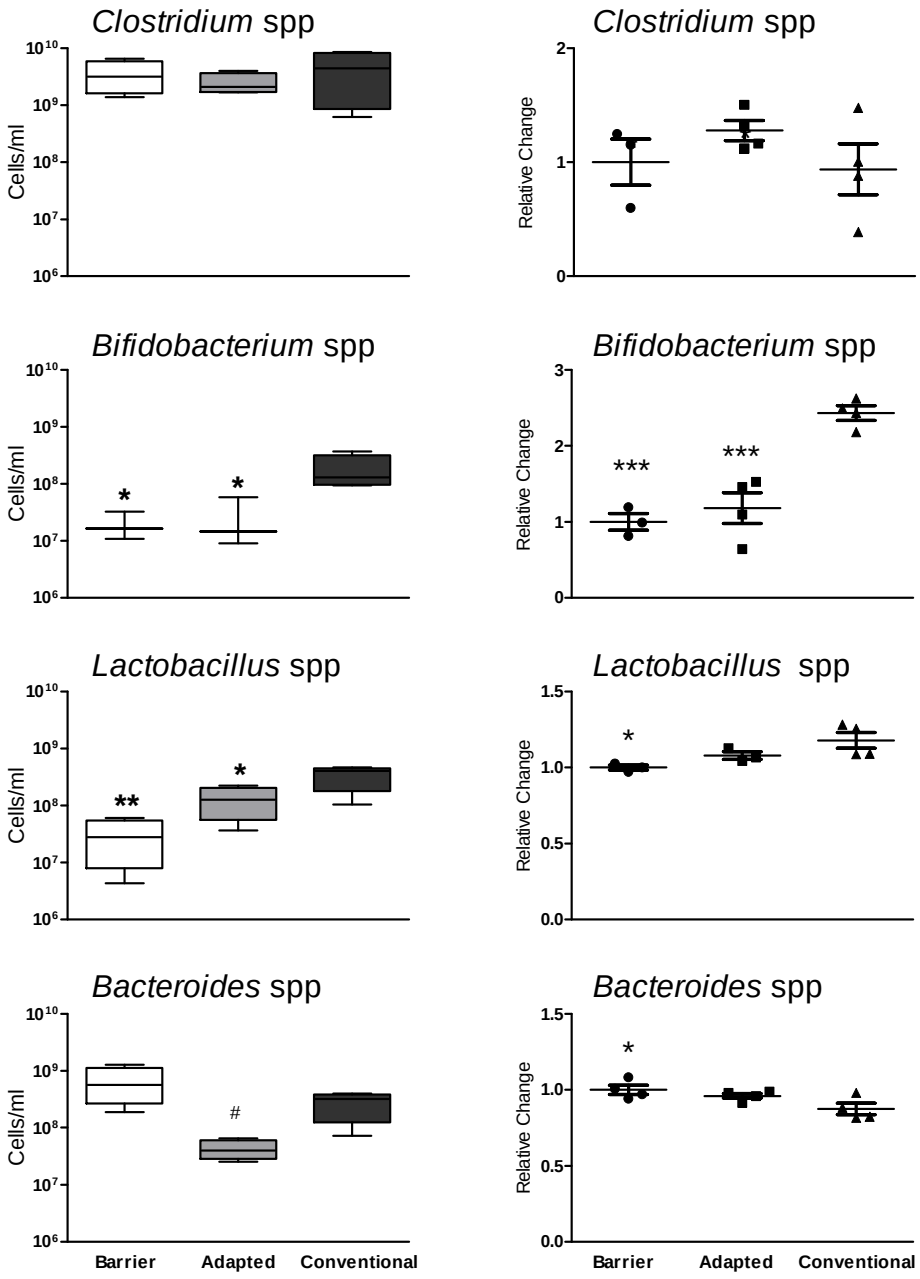


Figure 2. Comparison of the ceco-colonic microbiota, as quantified by FISH (left column) and qPCR (right column), in the same animals. FISH quantification (left column): data are media (interquartile range) ± SD for each bacterial group analyzed (n=4 per group). Because of technical problem one animal of the barrier group was not analyzed for Bifidobacterium spp. *, **: P < 0.05 or 0.01 vs. the conventional group. #: P<0.05 vs. the barrier group. qPCR quantification (right column): Each point represents an individual animal. The horizontal line with errors represent the mean ± SEM (n=4 per group). Because of technical problems one animal of the barrier group was not analyzed for Bifidobacterium spp. and Clostridium spp. and one animal of the adapted group was not analyzed for Bacteroides spp. *, ***: P < 0.05 or 0.001 vs. the conventional group. Barrier: barrier-breed-and-maintained group; Adapted: barrier-breed adapted to conventional conditions group; Conventional: conventional-breed-and-maintained group.

COLONIC EXPRESSION OF TLRS

TLRs expression was similar across groups, regardless the environmental conditions considered. Overall, colonic expression of TLR4 and 5 was similar across groups and higher (by 6-fold) than the expression of TLR2 or TLR7. Relative expression levels were TLR4 ~ TLR5 > TLR2 ~ TLR7 (Fig. 3).

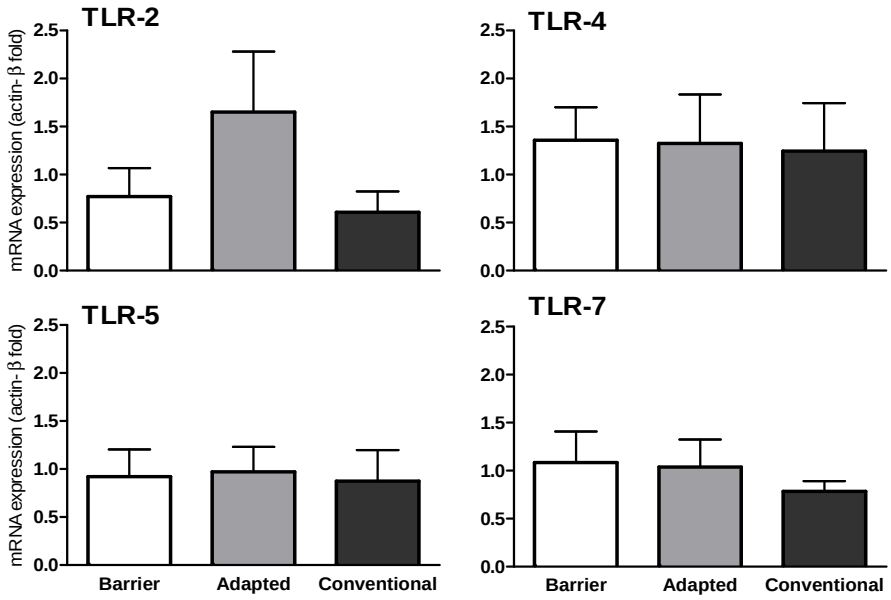


Figure 3. mRNA expression of TLR2, 4, 5 and 7 in colonic tissue samples of the different experimental groups. Barrier: barrier-breed-and-maintained group; Adapted: barrier-breed adapted to conventional conditions group; Conventional: conventional-breed-and-maintained group. Data are mean ± SEM of 4 animals per group.

COLONIC EXPRESSION OF SENSORY-RELATED MARKERS AND CORRELATION WITH BACTERIAL COUNTS

The most abundantly expressed sensory-related marker within the colon was PAR-2, with a minimum of 10-fold higher expression than other markers assessed, regardless the experimental group considered. In all cases, CGRP was undetectable. Other markers assessed, although detected in all samples analyzed, showed, in general, low levels of expression.

Expression of CB2 (P=0.1), PAR-2 (P=0.07), TRPV3 (P=0.03) and MOR (P=0.01) was higher in the barrier-breed-and-maintained group when compared to the conventional-breed-and-maintained group; showing intermediate levels of expression in the barrier-breed adapted to

conventional conditions group (Fig. 4). Expression levels of CB1 and TRPV1 and 4 were similar across groups.

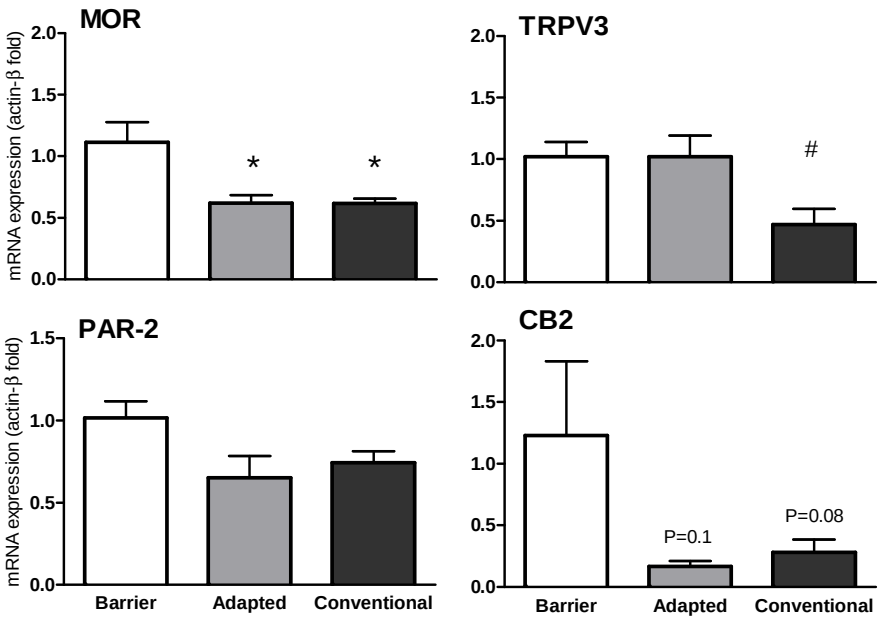


Figure 4. mRNA expression of MOR, TRPV3, PAR-2 and CB2 in colonic tissue samples of the different experimental groups. Barrier: barrier-breed-and-maintained group; Adapted: barrier-breed adapted to conventional conditions group; Conventional: conventional-breed-and-maintained group. Data are mean ± SEM of 4 animals per group. *: P < 0.05 vs. Barrier. #: P<0.05 vs. other groups. Barrier: barrier-breed-and-maintained group; Adapted: barrier-breed adapted to conventional conditions group; Conventional: conventional-breed-and-maintained group.

Expression levels of MOR and TRPV3 correlated with changes in the composition of the luminal microbiota. In particular, expression levels of TRPV3 showed a negative correlation with the counts of *Bifidobacterium* spp (P=0.01; r^2=0.55) and *Lactobacillus* spp. (P=0.02; r^2=0.45) (Fig. 5). Similarly, Enterobacteria counts also tended to correlate in a negative manner with TRPV3 mRNA (P=0.07; r^2=0.27). On the other hand, expression levels of MOR showed a clear tendency to positively correlate with the counts of *Bacteroides* spp. (P=0.07; r^2=0.28) and to negatively correlate with *Lactobacillus* spp. counts (P=0.07; r^2=0.27; Fig. 5). Similar correlations were determined when using relative bacterial abundance determined by qPCR (data not shown).

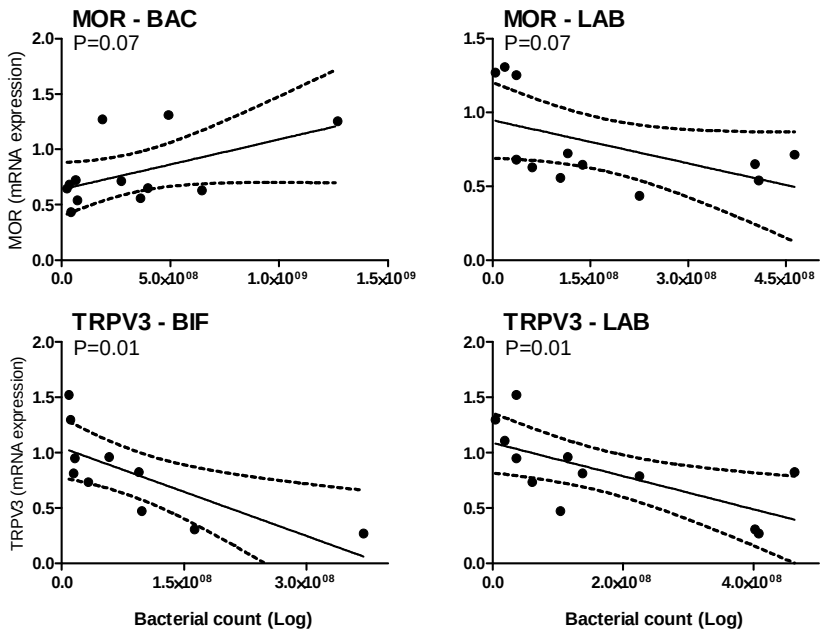


Figure 5. Correlation between the relative expression of sensory-related markers and bacterial counts. Up: MOR and down: TRPV3 for either Bacteroides spp. (BAC), Lactobacillus - Enterococcus spp. (LAB) or Bifidobacterium spp. (BIF). Each point represents an individual animal. Broken lines represent the 95% confidence interval.

LOCALIZATION AND QUANTIFICATION OF CANNABINOID AND μ OPIOID RECEPTORS BY IMMUNOHISTOCHEMISTRY

CB2 immunoreactivity was observed in the luminal surface of the epithelial microvilli and within ganglionic cells of the myenteric plexus. Very few immunoreactive cells were observed in the submucous plexus. Intense CB2 immunoreactivity was observed in Peyer's patches and also in some unidentified cells distributed through the submucosa, likely corresponding to immune cells. The smooth muscle of the blood vessels walls showed intense immunoreactivity. Overall, similar patterns of distribution and staining intensities were observed in all experimental groups (Fig. 6). Nevertheless, CB2-immunoreactive ganglionic cell density within the myenteric plexus had a clear trend to be lower in the conventional-breed-and-maintained group compared with the other experimental groups, although statistical significance was not achieved (P=0.053; Fig. 6).

Immunostaining for CB1 receptors was found in epithelial cells, mainly within the apical surface of the microvilli. In the submucosa, a fainter positive staining was seen in the smooth

muscle of the blood vessel walls. No staining was observed in the muscle layers. Cells within ganglia of the myenteric plexus appeared also immunostained, while within the submucous plexus immunoreactive cells were rarely seen. Within the myenteric plexus, the density of immunoreactive cells was low: CB1–immunoreactive cells oscillated between 0 and 2 cells/ganglion and was similar among all experimental groups (data not shown). Peyer's patches showed a moderate staining in well-defined cells. Similar distribution and staining intensities were observed in all experimental groups (data not shown).

MOR immunoreactivity was mainly found within the ganglia of both myenteric and submucosal plexuses. Some epithelial cells as well as unidentified cells in the lamina propia appeared also stained (Fig. 6). Immunostaining intensity within the myenteric plexus was higher in the barrier-breed-and-maintained group and showed a clear tendency to decrease with the adaptation to conventional housing conditions (Fig. 5).

In all cases, absence of the primary antibody resulted in a complete loss of immunoreactivity, thus confirming the specificity of the staining.

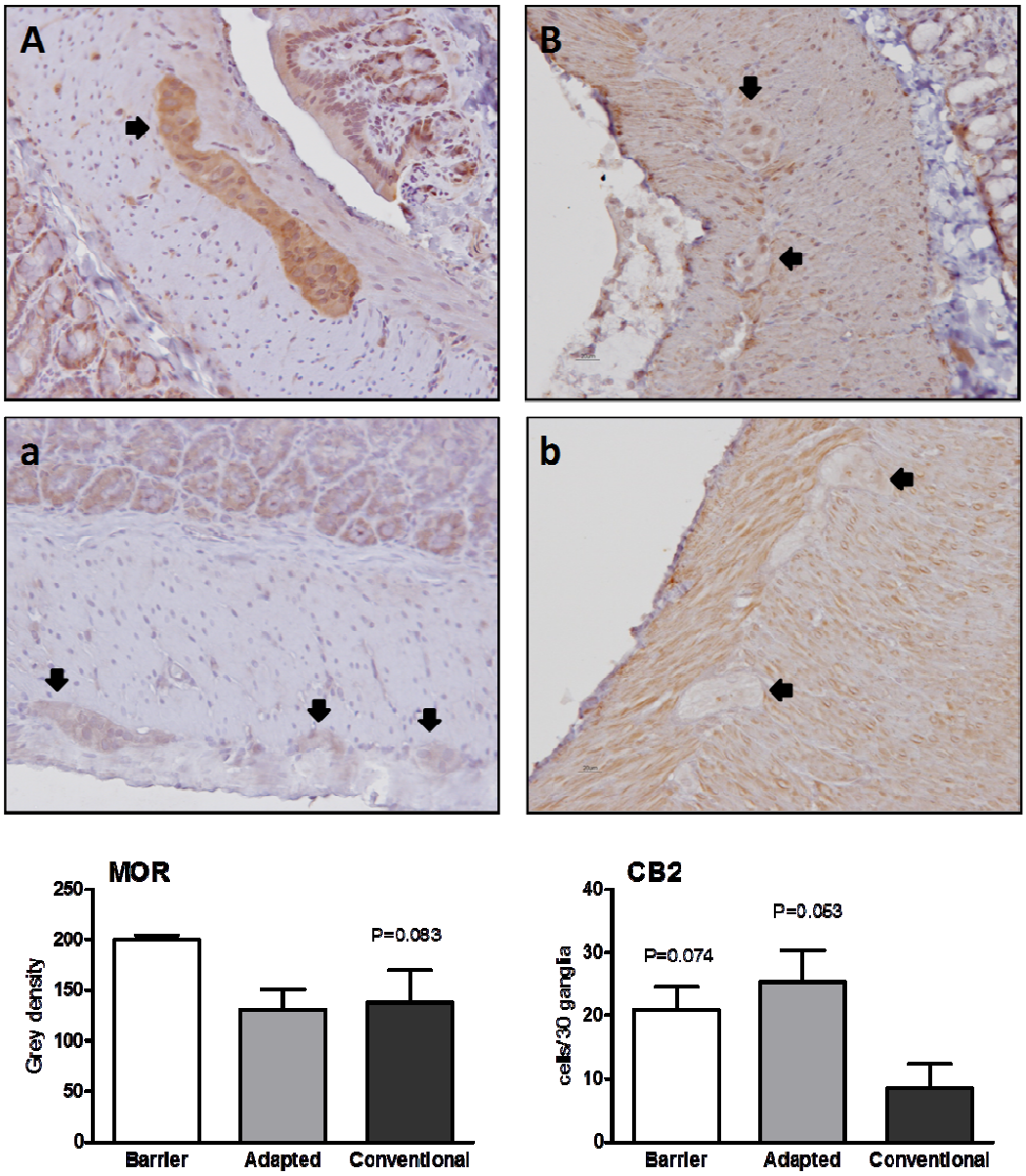


Figure 6. Expression of MOR and CB2 assessed by immunohistochemistry. Representative images (X200) showing localization of MOR (A, a) and CB2 receptor (B, b) immunoreactivity in the myenteric plexus of the rat colon. Upper panels (A, B) correspond to a barrier-breed-and-maintained animal; lower panels (a, b) correspond to a conventional-breed-and-maintained animal. Arrows indicate myenteric ganglia. Bar graphs at the bottom show the quantification of MOR-like immunoreactivity in the myenteric ganglia and the number of CB2-immunorreactive cells within the myenteric plexus in the different experimental groups. Barrier: barrier-breed-and-maintained group; Adapted: barrier-breed adapted to conventional conditions group; Conventional: conventional-breed-and-maintained group. Data are mean ± SEM (n=4 per group). Number on top of columns corresponds to the P values (ANOVA) vs. barrier for MOR and vs. conventional for CB2. Barrier: barrier-breed-and-maintained group; Adapted: barrier-breed adapted to conventional conditions group; Conventional: conventional-breed-and-maintained group.

DISCUSSION

During the last years there has been an increasing interest in the role of GCM in the maintenance of gastrointestinal homeostasis and its potential implication in pathophysiological conditions. Here we present, for the first time, evidence that spontaneous variations in gut microbiota, related to environmental adaptation, are associated with changes in the expression of secretomotor and sensory-related markers within the gut (mainly the opioid, vanilloid and endocannabinoid systems), without alterations in TLRs expression.

We show that the environmental conditions of breeding/housing of the animals determine, at least partially, the composition of the GCM, as previously shown for both rats and mice.[12–14] Moreover, in agreement with our previous observations [12], results obtained indicate that relative composition of the ceco-colonic microbiota in rats varies spontaneously with changes in the environmental conditions. Changes in the composition of GCM observed here are in concordance with those previously described by us in similar experimental conditions and using animals of the same genetic background.[12] Main microbial changes associated to the adaptation from a barrier to a conventional environment implied a loss of *Bacteroides* spp. with a simultaneous increase in the counts of *Lactobacillus-Enterococcus* spp. and *Bifidobacterium* spp. Quantitative changes in bacterial composition across groups were further confirmed using qPCR. Overall, relative bacterial abundances assessed by qPCR or direct bacterial quantifications by FISH were equivalent. Since qPCR is a more sensitive technique than FISH, these similarities reinforce the value of our FISH data and indicate that the results described reflect a consistent change in microbial composition.

Numerous reports have implicated GCM in the development of immune responses within the gut and identified bacterial flora as a significant component of the pathogenesis of several intestinal disorders, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).[2–5] Following these observations, several studies suggest that microbiota might influence immune- and neurally-mediated responses within the gut. In particular, several lines of evidence suggest that bacterial flora is implicated in the modulation of sensory mechanisms arising from the gut and, therefore, in changes in visceral pain-related responses. First, clinical and preclinical studies have shown that administration of certain probiotics can prevent abdominal symptoms in IBS and IBD patients (pain and discomfort, bloating or altered bowel habits).[6,8,27–31] Second, probiotic-like treatments reduced visceral pain-related responses in animal models of the

disease.[7,10] Despite the clinical interest of these observations the mechanisms mediating these beneficial effects remain largely unknown. Animal data suggest, at least, a microbial-dependent modulation of the opioid and the endocannabinoid systems, both mediating analgesic responses within the gut[16,18,19], as a potential underlying mechanism.[10,11] Here we show that, in rats, spontaneous adaptive variations of GCM, associated to environmental changes, lead to a modulation of the expression of sensory-related systems within the gut, including the opioid, the endocannabinoid and the vanilloid (TRPV) systems. Altogether, these data support a role for microbiota affecting sensory-related gastrointestinal regulatory mechanisms.

Preliminary observations suggest that specific bacterial strains might modulate the endogenous expression of several mediators implicated in viscerosensitivity. For instance, a specific strain of *Lactobacillus acidophilus* given as a probiotic was able to modulate the content of CB2 and MOR in the gut and to reduce visceral pain responses in rats.[10] These results agree with the present observations showing that spontaneous adaptive variations of the GCM imply changes in the intestinal expression of receptors implicated in sensory/nociceptive-related mechanisms. In the present study we did not correlate changes in receptors expression with any particular strain of bacteria, but with large spontaneous changes in gut commensal microbiota. From our observations, spontaneous fluctuations of *Bacteroides* spp, *Bifidobacterium* spp. and *Lactobacillus* spp strains correlate with changes in sensory-related markers. Although no clear cause-effect relationship can be inferred, these observations suggest that such bacterial strains might be (directly or indirectly) implicated in the changes observed in sensory-related markers. Previous studies have pointed towards two main genera of bacteria as having beneficial effects in gut homeostasis, including the modulation of visceral pain-related responses: *Lactobacillus* spp and *Bifidobacterium* spp.[6–8,10,27,29,30] In our conditions, spontaneous changes in these bacterial groups correlated with variations in TRPV3 and MOR expression. This partially agrees with the previous reports in which gut microbiota was related with the content of MOR and CB2 receptors in the gut, leading to a state of analgesia-like.[10] Interestingly, expression changes were observed in both pro-nociceptive (TRPV3 and PAR-2) and anti-nociceptive (MOR and CB2) systems. In all cases, a down-regulation was observed in the adaptation process from barrier to conventional conditions. This might suggest differences in pain responses between animals maintained in barrier or standard conditions or during the adaptation process from one environment to the other; although this remains to be demonstrated. Supporting this view, large alterations of gut microbiota, associated to the treatment with antibiotics or by adaptation to a standard, non-sterile environment, was

associated to altered visceral pain responses in mice.[32,33] Moreover, mice data suggest also that GCM is fundamental for the development of inflammatory pain.[34] Therefore, it is feasible to assume that microbial changes may result in alterations in visceral pain responses.

It is important to remind that the receptor modulation was initially determined at the gene expression level (mRNA). To further determine if expression changes could have consequences at the protein level we further assessed the expression of CB1/CB2 and MOR in colonic tissues using immunohistochemistry. In all cases CB1, CB2 and MOR immunoreactivity were identified throughout the colon. Tissue distribution observed for either receptor was in accordance to that previously described for the colon in mice, rats and pigs.[35–37] Overall, no differences among groups in immunoreactivity were observed for none of the receptors assessed. Nevertheless, a more detailed analysis of immunoreactivity in the myenteric plexus indicated lower protein content in ganglionic structures in conventional-breed-and-maintained animals compared with the barrier-breed-and-maintained group; in agreement with the mRNA expression results. This reinforces the view that these changes might translate into functional differences in CB2- and MOR-mediated responses.

As mentioned, the mechanisms through which GCM influences the expression of neuro-immune mediators remain largely unknown. Extensive work has demonstrated that the microbiota interacts with the internal milieu through specific bacterial recognition systems. These systems, with TLRs as main exponent, recognize bacterial components throughout specific epithelial receptors.[9,20–23] We previously showed that spontaneous adaptive variation of the gut microbiota are associated to minor changes in bacterial recognition systems, in particular TLR2 and TLR4 expression; and that the expression of these TLRs had no clear correlation with the microbiota.[12] The present results agree with these observations and show that spontaneous changes of GCM are not enough to directly modify the expression of TLR2, 4, 5 or 7. It can be speculated that profound changes in the microbiota, including the appearance of pathogenic strains, can be necessary to significantly alter host-microbial interactions, leading to changes in the expression of TLRs.

Overall, the present observations support the view that GCM is a dynamic system able to experience environmental-related adaptive changes. Moreover data obtained suggest that the microbiota is able to interact with the host leading to the modulation of endogenous regulatory systems. This might be part of the underlying mechanisms mediating the beneficial effects of

certain probiotics on gastrointestinal disorders. The present data directly implicate large spontaneous changes in gut commensal microbiota with the modulation of endogenous systems potentially implicated in sensory responses arising from the gut. These observations warrant further studies assessing how spontaneous or directed changes in gut commensal flora affect neural functions within the gut from a functional, morphological and molecular point of view.

ACKNOWLEDGEMENTS

We thank Emma Martínez and Antonio Acosta for their technical support in different stages of the study. This work was supported by grants BFU2009-08229 from the Spanish *Ministerio de Ciencia e Innovación* and 2009SGR-708 from the *Generalitat de Catalunya*. M. Aguilera personal support from the FPI program (BES-2010-037699 - Spanish *Ministerio de Ciencia e Innovación*).

REFERENCES

1 Camp JG, Kanther M, Semova I, Rawls JF. Patterns and scales in gastrointestinal microbial ecology. Gastroenterology 2009;136:1989–2002.

2. Kelly D, Mulder IE. Microbiome and immunological interactions. Nutr Rev 2012;70 Suppl 1:S18–30.

3. Collins SM, Bercik P. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology 2009;136:2003–14.

4. Looijer-van Langen MAC, Dieleman LA. Prebiotics in chronic intestinal inflammation. Inflamm Bowel Dis 2009;15:454–462.

5. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427–434.

6. Diop L, Guillou S, Durand H. Probiotic food supplement reduces stress-induced gastrointestinal symptoms in volunteers: a double-blind, placebo-controlled, randomized trial. Nutr Res 2008;28:1–5.

7. Eutamene H, Lamine F, Chabo C, Theodorou V, Rochat F, Bergonzelli GE, et al. Synergy between Lactobacillus paracasei and its bacterial products to counteract stress-induced gut permeability and sensitivity increase in rats. J Nutr 2007;137:1901–1907.

8. Fan Y, Chen S, Yu Y, Si J, Liu B. A probiotic treatment containing Lactobacillus, Bifidobacterium and Enterococcus improves IBS symptoms in an open label trial. J Zhejiang Univ Sci B 2006;7:987–991.

9. Honda K, Takeda K. Regulatory mechanisms of immune responses to intestinal bacteria. Mucosal Immunol 2009;2:187–196.

10. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 2007;13:35–37.

11. Verdú EF, Bercik P, Verma-Gandhu M, Huang X-X, Blennerhassett P, Jackson W, et al. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 2006;55:182–190.

12. Terán-Ventura E, Roca M, Martin MT, Abarca ML, Martinez V, Vergara P. Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats. Microb Ecol 2010;60:691–702.

13. Campbell JH, Foster CM, Vishnivetskaya T, Campbell AG, Yang ZK, Wymore A, et al. Host genetic and environmental effects on mouse intestinal microbiota. ISME J 2012;6:2033–44.

14. Ma BW, Bokulich NA, Castillo PA, Kananurak A, Underwood MA, Mills DA, et al. Routine habitat change: a source of unrecognized transient alteration of intestinal microbiota in laboratory mice. PLoS One 2012;7:e47416.

15. Blackshaw LA, Brierley SM, Hughes PA. TRP channels: new targets for visceral pain. Gut 2010;59:126–135.

16. Brusberg M, Arvidsson S, Kang D, Larsson H, Lindström E, Martinez V. CB1 receptors mediate the analgesic effects of cannabinoids on colorectal distension-induced visceral pain in rodents. J Neurosci 2009;29:1554–1564.

17. Bueno L. Protease activated receptor 2: a new target for IBS treatment. Eur Rev Med Pharmacol Sci 2008;12 Suppl 1:95–102.

18. Davis MP. Drug management of visceral pain: concepts from basic research. Pain Res Treat 2012;2012:265605.

19. Izzo AA, Sharkey KA. Cannabinoids and the gut: new developments and emerging concepts. Pharmacol Ther 2010;126:21–38.

20. Cario E. Therapeutic impact of toll-like receptors on inflammatory bowel diseases: a multiple-edged sword. Inflamm Bowel Dis 2008;14:411–421.

21. Carvalho FA, Aitken JD, Vijay-Kumar M, Gewirtz AT. Toll-like receptor-gut microbiota interactions: perturb at your own risk! Annu Rev Physiol 2012;74:177–198.

22. Marques R, Boneca IG. Expression and functional importance of innate immune receptors by intestinal epithelial cells. Cell Mol Life Sci 2011;68:3661–3673.

23. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229–241.

24. Harmsen HJM, Raangs GC, He T, Degener JE, Welling GW. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl Environ Microbiol 2002;68:2982–2990.

25. Selinummi J, Seppälä J, Yli-Harja O, Puhakka JA. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 2005;39:859–863.

26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408.

27. Dinoto A, Suksomcheep A, Ishizuka S, Kimura H, Hanada S, Kamagata Y, et al. Modulation of rat cecal microbiota by administration of raffinose and encapsulated Bifidobacterium breve. Appl Environ Microbiol 2006;72:784–792.

28. Kajander K, Hatakka K, Poussa T, Färkkilä M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. Aliment Pharmacol Ther 2005;22:387–394.

29. Whelan K. Probiotics and prebiotics in the management of irritable bowel syndrome: a review of recent clinical trials and systematic reviews. Curr Opin Clin Nutr Metab Care 2011;14:581–587.

30. Wildt S, Munck LK, Vinter-Jensen L, Hanse BF, Nordgaard-Lassen I, Christensen S, et al. Probiotic treatment of collagenous colitis: a randomized, double-blind, placebo-controlled trial with Lactobacillus acidophilus and Bifidobacterium animalis subsp. Lactis. Inflamm Bowel Dis 2006;12:395–401.

31. Williams EA, Stimpson J, Wang D, Plummer S, Garaiova I, Barker ME, et al. Clinical trial: a multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study. Aliment Pharmacol Ther 2009;29:97–103.

32. Veerappan GR, Betteridge J, Young PE. Probiotics for the treatment of inflammatory bowel disease. Curr Gastroenterol Rep 2012;14:324–333.

33. Aguilera M, Vergara P, Martínez V. Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. Neurogastroenterol Motil 2013;25:e515–529.

34. Verdu EF, Collins SM. Irritable bowel syndrome and probiotics: from rationale to clinical use. Curr Opin Gastroenterol 2005;21:697–701.

35. Amaral FA, Sachs D, Costa V V, Fagundes CT, Cisalpino D, Cunha TM, et al. Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 2008;105:2193–2197.

36. Coutts AA, Irving AJ, Mackie K, Pertwee RG, Anavi-Goffer S. Localisation of cannabinoid CB(1) receptor immunoreactivity in the guinea pig and rat myenteric plexus. J Comp Neurol 2002;448:410–422.

37.Holzer P. Opioid receptors in the gastrointestinal tract. Regul Pept 2009;155:11–27.

38. Wright KL, Duncan M, Sharkey KA. Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation. Br J Pharmacol 2008;153:263–270.

39. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, et al. Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PLoS One 2011;6:e28654.

40. Haarman M, Knol J. Quantitative real-time PCR analysis of fecal Lactobacillus species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 2006;72:2359–2365.

CHAPTER 2

STIMULATION OF COLONIC TOLL-LIKE RECEPTORS LEADS TO A LOCAL IMMUNE AND SENSORY-RELATED ACTIVATION WITH MINOR CHANGES IN THE COMMENSAL MICROBIOTA IN RATS

M. Aguilera[a,c], J. Pla[a], V. Martínez[a,b,c]

[a]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain.

[b]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Spain.

[c]Neuroscience Institute, Universitat Autònoma de Barcelona, Barcelona, Spain.

ABSTRACT

Toll-like receptors (TLRs) participate in microbial recognition within the gut. Dysbiotic states might generate an imbalance in TLR-mediated signaling leading to exaggerated immune responses and the development of intestinal inflammation and sensory motor alterations. We characterized local neuro-immune responses and changes in sensory-related systems associated to the selective stimulation of colonic TLR4 (LPS, *E. coli* O5:B55) or TLR7 (imiquimod) in rats.

A time-related (single vs. 5-day repeated treatment) and TLR-specific response was observed. Overall, LPS-mediated stimulation of TLR4 lead to higher responses in magnitude; further enhanced during a 5-day repeated treatment vs. a single treatment. Stimulation of TLR4 lead to significant up-regulation of inflammatory markers with changes in host-bacterial interaction systems, including up-regulation of TLRs, integrins and antimicrobial peptides, and an increase in the ratio of secretory-IgA-coated bacteria. Sensory-related markers (cannabinoid receptors, TRPV1/3/4 and CGRP) were also up-regulated by LPS. Imiquimod had only marginal effects. Luminal microbiota was not affected, although LPS enhanced epithelial adherence for Clostridia/Bifidobacteria. No histopathological alterations consisting with the induction of inflammation were observed.

Stimulation of colonic TLRs by microbial-related luminal factors elicits a TLR-specific immune activation, without inflammatory-like structural changes, and modulates the expression of sensory-related systems. Changes observed might be part of the host's response to an alteration of the microbiome, with the objective of regaining a balance within the commensal microbiota, avoiding excessive immune responses and the development of inflammation. Similar mechanisms might explain the beneficial/detrimental effects described for different bacterial strains and might represent a basis for the mechanism of action of probiotics.

Keywords: gut commensal microbiota, toll-like receptors, imiquimod, lipopolysaccharide, visceral pain, intestinal neuro-immune interactions, microbiota-host response

Chapter 2

INTRODUCTION

During the last years, gut commensal microbiota (GCM) has been recognized as a key element in gastrointestinal homeostasis.[1–3] Consequently, states of dysbiosis, with altered host-bacterial interactions, have been associated with several gastrointestinal and extra-intestinal pathophysiological states.[4] The host-dependent recognition and interaction with the microbiota seem to be a key component in this process, leading to immune-related signaling cascades that would result ultimately in the modulation of neural and endocrine processes and the generation of inflammation and functional alterations at various levels (both inside and outside the GI tract).[1,3,5,6]

Within the GI tract, host-bacterial interactions are largely mediated by Toll-like receptors (TLRs). TLRs are a family of transmembrane glycoproteins that belong to the innate immune system. They are located on the cell surface or in endosomes and recognize microbes through pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).TLRs expression on intestinal epithelial cells (IECs) is low and has a predominant basolateral distribution, thus reducing the interaction with luminal commensal PAMPs. Nevertheless, certain TLR ligands and the TLR-mediated recognition of commensal microorganims induce a signaling response (cytokines- and chemokines-mediated) that protects the host from potential pathogens and, at the same time, prevents exaggerated immunity to commensals.[7–10] At the same time, in states of altered microbiota, TLRs will recognize the dysbiotic situation and will generate local immune responses towards the abnormal microbiota with the finality of preventing bacterial translocation and aiming to restore the normal microbial status. However, in certain situations, the same system can generate exaggerated immune responses leading to the development of intestinal inflammation.[7,9] In these situations, TLRs-mediating signaling cascades might also affect neuro-endocrine components generating functional alterations within the GI tract. For instance, others and us have shown that states of colonic dysbiosis are associated to changes in the expression of sensory-related markers and altered viscerosensitivity in mice and rats.[11–14] Similar modulatory actions are likely to explain the beneficial effects of certain probiotics in intestinal inflammation and visceral pain.[15–17]

Taking into account the large complexity of the GCM, the multiple changes that it can experience during dysbiotic states and the fact that TLRs are able to recognize a large array of bacterial products [18–20] we explored changes in host-bacterial interactions and the local immune and sensory-related responses elicited by the selective stimulation of colonic TLRs, in particular

TLR4 and TLR7. Both, TLR4 and TLR7 are expressed in the intestine and have been implicated in microbial-related pathophysiological states. TLR4 is highly expressed on colonocytes [9,20–22] while TLR7 is mainly present on dendritic cells (DCs), with both being found in enteric neurons.[9,21] Their role might differ in different pathophysiological conditions. For instance, while both are up-regulated in GI inflammatory conditions [23,24] TLR4 is up-regulated and TLR7 down-regulated in patients with irritable bowel syndrome (IBS) or in some animal models of intestinal dysbiosis.[25] Since there is a bidirectional communication between the host and the microbiota, it is feasible to assume that microbial-derived signals will elicit feedback responses in the host directed towards the modulation of the microbiota. Therefore, we also assessed if the selective stimulation of colonic TLR4 and TLR7 translates into changes of the luminal and wall-adhered microbiota.

Materials and Methods

Animals

Adult male (8-9 weeks-old) OFA Sprague Dawley rats (Charles River Laboratories, Lyon, France) were used. Upon arrival, animals were acclimatized for a 1-week period prior to any experimentation. All animals were maintained in standard conditions in an environmentally controlled room (20-22ºC, 12 h light:dark cycle), with food and water *ad libitum*. All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocol 1101) and the Generalitat de Catalunya (protocol 5645).

Drug administration

For stimulation of TLR7 and TLR4 the ligands Imiquimod (selective TLR7 agonist; 4-Amino-1-isobutyl-1H-imidazo(4,5-c) quinoline; ALX-420-039, Enzo Life Sciences, New York, USA) and lipopolysaccharide from Escherichia coli 055:B5 (selective TLR4 ligand; LPS; L4524, Sigma-Aldrich, St. Louis, Missouri, USA) were used. Compounds were administered topically into the colon. For this, rats were anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and a flexible plastic cannula (length, 7.5 cm; diameter, 0.61mm) was introduced through the anus to reach the mid-colon (4 cm from the anus). Thereafter compounds or their vehicles (0.02 mL) were administered

slowly (over a 1-2 min period). To avoid backflow of the solutions, animals were maintained anesthetized in an inclined position for an additional 2-4 min period.

EXPERIMENTAL PROTOCOLS

Rats (n=40) were randomly divided into 8 experimental groups depending upon the treatments applied (Table 1). Animals received a single or a 5-days repeated intracolonic treatment of imiquimod (0.2 mg/animal/administration), LPS (0.1 mg/animal/administration) or their vehicles (0.5% hydroxy-propyl-methyl cellulose in sterile saline or saline, respectively; 0.2 mL/animal/administration). Doses were selected based on pilot studies. All treatments were applied in the morning, between 8:00 and 9:00 h. In all cases, 24 h after the last administration, animals were euthanized and tissue and fecal samples obtained (see below).

Table 1. Experimental groups.

Treatment	Intracolonic Dose	Duration	N
Vehicle - sterile saline	0.2 mL/administration	1 administration	4
		5 administrations	4
TLR4 agonist - LPS	0.2 mg/0.2 mL administration	1 administration	6
		5 administrations	6
Vehicle - 0.5% hydroxy-propyl-methyl cellulose (HPMC) in sterile saline	0.2 mL/administration	1 administration	4
		5 administrations	4
TLR7 agonist - Imiquimod	0.2 mg/0.2 mL administration	1 administration	6
		5 administrations	6

CLINICAL SIGNS

Clinical and macroscopic assessment of the animals included daily monitoring of body weight and body temperature, appearance of faeces and general health condition. Separate scores (0–2) were assigned for faecal consistency and health condition (including hunch posture and piloerection); where 0 indicates normal faecal content/healthy condition, 1 indicates loose faecal content/signs of hunch posture and/or piloerection and 2 indicates watery diarrhoea/ severe hunch posture and piloerection. At necropsy (see below), the macroscopic appearance of the

colon was scored, in a blind manner, based on the presence of inflammatory signs (oedema,thickness, stiffness and presence of ulcerations), consistency of faecal content and presence of visible faecal blood; as previously published.[26]

SAMPLES COLLECTION

24 h after the last administration animals were deeply anesthetized with Isoflurane (Isoflo) and euthanized by intracardiac exsanguination followed by thoracotomy; thereafter, a medial laparotomy was performed, the ceco-colonic region localized and the cecum and colon dissected. Subsequently, ceco-colonic fecal contents and a tissue sample from the middle colon were collected and frozen immediately in liquid nitrogen. Frozen samples were stored at –80 ºC until analysis. At the same time, tissue samples of the proximal colon (about 1.5 cm each) were collected and fixed overnight at 4ºC in Carnoy fixative (ethanol:chloroform:glacial acetic acid, 6:3:1, v:v:v) or in 4% paraformaldehyde. After an overnight fixation, tissues were paraffin embedded and 5µm thick sections obtained. In addition, the adrenal glands, the thymus and the spleen were dissected and weighted.

BACTERIAL IDENTIFICATION BY FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

For fluorescence *in situ* hybridization (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; Isogen, Barcelona, Spain). Probes used were: EUB 338 (5'GCTGCCTCCCGTAGGAGT3') to total Bacteria; NON 338 (5'ACATCCTACGGGAGGC3') to non-bacteria (negative control); BAC 303 (5'CAATGTGGGGGACCTT3') to *Bacteroides* spp.; EREC 482 (5'GCTTCTTAGTCAGGTACCG3') to *Clostridium* Cluster XIVa; LAB 158 (5'GGTATTAGCACCTGTTTCCA3') to *Lactobacillus* spp. and *Enterococcus* spp.; ENT-D (5'TGCTCTCGCGAGGTCGCTTCTCTT3') to Enterobacteria; BIF 164 (5'CATCCGGCATTACCACCC3') to *Bifidobacterium* spp. and MUC-1437 (5'CCTTGCGGTTGGCTTCAGAT 3') to Verrucobacteria.

Fecal samples of ceco-colonic content and colonic carnoy fixed tissue were used to characterize luminal and wall-adhered microbiota, respectively, following techniques previously described by us.[12] Hybridized slides were viewed under oil immersion, using a Carl Zeiss Axioskop 40 FL epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (Zeiss

AxioCam MRm) for obtaining digital images (Zeiss AxioVision Release 4.8.1). For quantification of luminal bacteria, 20 randomly selected fields were photographed, the number of hybridized cells counted using the CellC software.[27] and the mean value obtained. 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 15% of the pictures observed (3 out of 20) was required to describe bacterial attachment to the epithelium. All procedures were performed on coded slides, to avoid bias.

mRNA ANALYSIS

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit, (AM1924M, Ambion, California, USA) and FastRNA Pro Green Kit (6045-050, MP Biomedicals, Solon, OH). RNA was quantified with NanoDrop (ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA);a consistent 260/280 ratio (between 1.8-2) was considered acceptable to perform a two-step quantitative real time PCR (RT-qPCR)using hydrolysis probes (TaqMan Assays). RNA samples were converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA). TaqMan gene expressions assays for cannabinoid receptors type 1 (CB1; Rn00562880_m1) and 2 (CB2; Rn00571953_m1), fatty acid amide hydrolase (FAAH; Rn00577086_m1), μ-opioid receptor (Rn01430371_m1), transient receptor potential vanilloid 1 (TRPV1; Rn00583117_m1), 3 (TRPV3; Rn01460303_m1) and 4(TRPV4; Rn01460303_m1), proteinase-activated receptor type 2 (PAR-2; Rn00588089_m1), calcitonin gene-related peptide (CGRP; Rn01511354_m1), interleukin 6(IL-6; Rn01410330_m1), interferon α1 (IFNα1; Rn02395770_g1), interleukin 10 (IL-10; Rn00563409_m1), interleukin 12p40 (IL-12p40; Rn00575112_m1), Toll-like receptor 2 (TLR2; Rn02133647_s1), 4 (TLR4; Rn00569848_m1), 5 (TLR5; Rn01411671_s1) and 7 (TLR7; Rn01771083_s1), integrin α2 (Itg α2; Rn01489315_m1) and β1 (Itg β1; Rn00566727_m1), Defensin α24 (Def α24; Rn02769344_s1) and β4 (Def β4; Rn01756705_m1), regenerating islet-derived 3 γ (Reg3γ; Rn00687437_g1) and Resistin-like molecule β (RELMβ; Rn01439306_m1) were used (all from Applied Biosystems). Actin-β (Rn00667869_m1) was used as endogenous reference gene.

The PCR reaction mixture was incubated on the Applied Biosystems 7500 FastReal Time PCR System. All samples, as well as the negative controls, were assayed in triplicates. The cycle threshold for each sample was obtained and thereafter, all data were analyzed with the comparative Ct method (2− ΔΔCt) with the control group serving as the calibrator.[28]

Quantification of secretory immunoglobulin A and IgA-coated bacteria

Luminal IgA secretion was measured in fresh homogenates of cecal contents (equally diluted in PBS 1x) using a commercial double-antibody sandwich ELISA, following manufacturer's instructions (MBS564117; MyBiosource, San Diego, USA).

For quantification of IgA coated-bacteria the same fresh homogenates were used. Bacteria were separated by centrifugation and a rabbit anti-Rat IgG/IgM/IGA Secondary Antibody, FITC conjugate (SA1-36148; Fisher Cientific) was used following described protocols, with minor modifications.[29] Samples were stained and mounted in slides with Vectashield Mounting Medium with DAPI (H-1200; VectorLabs, Peterborough, UK).20 randomly selected fields (per sample and in duplicate) were photographed using a Carl Zeiss Axioskop 40 FL epifluorescence microscope and used to count labeled bacteria.

Histology

For histological examination, hematoxylin-eosin-stained sections were obtained from paraffin-fixed tissue samples following standard procedures. A histopathological score (ranging from 0, normal, to 9, 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), 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), and presence of inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate). Histopathological scoring was performed on coded slides by two independent researchers.

The number of goblet cells was quantified in Carnoy-fixed samples of colonic tissue. Tissue sections were stained with Alcian Blue pH 2.5/Periodic Acid Schiff (AB 2.5/PAS kit; Bio-Optica, Milano, Italy) in order to specifically stain neutral (pink) and acidic (blue) mucins. Thereafter,

colonic goblet cells were counted in 20 longitudinally-oriented villus-crypt units. Length of the villus-crypt unit was also determined to obtain goblet cells density (number of cellsmm^{-1}).All measurements were performed on coded slides using the Zeiss AxioVision Release 4.8.1 software.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Data were analyzed by a two-way analysis of variance (ANOVA), taking treatment and duration of treatment as factors, followed, when necessary, by a Student-Neuwman-Keuls multiple comparisons test. A Chi-square test was used to analyze bacterial adherence. Data were considered statistically significant when $P< 0.05$.

RESULTS

Overall, all parameters assessed were similar in saline- or HPMC-treated animals, therefore, for the sake of clarity, these two groups have been merged in a single control group (identified as a vehicle group, for either the single or the 5-day repeated treatment).

Body weight changes were similar across experimental groups (Fig. 1A). Similarly, regardless the group considered, no clinical signs were observed after either the single or the repeated treatment (data not shown).

MACROSCOPIC AND MICROSCOPIC EVALUATION OF THE COLON AND WEIGHT OF BODY ORGANS

At necropsy, regardless the treatment applied, no macroscopic or microscopic signs of colonic inflammation were observed (Figs. 1B and 1D). Similarly, no differences across groups were observed in goblet cell density. Nevertheless, the number of goblet cells containing a mixture of acid and neutral mucins was increased after a single dose of LPS (6.9±1.1 cells/crypt; vehicle: 2.0±1.4 cells/crypt; $P<0.05$; Fig. 1C), coinciding with a slight reduction in the number of cells containing acidic mucins. Length of the colonic crypts was similar across groups.

Relative weight of thymus, spleen and adrenal glands was similar across groups (data not shown).

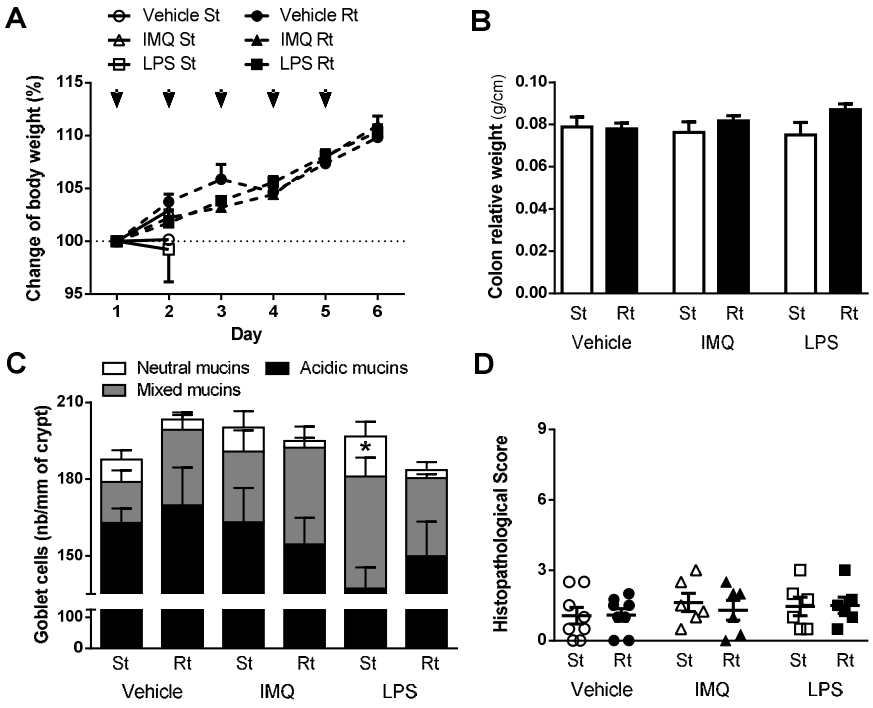


Figure 1. Clinical and histological signs associated to the different treatments. A: Changes in body weight. Arrows indicate the times of intracolonic treatment (single, open symbols; or repeated for 5 days, closed symbols). Data are mean±SEM. B: Relative weight of the colon at the time of necropsy. Data are mean±SEM. C: Goblet cells density according to the type of mucins expressed (neutral, acidic or mixed). Data are mean±SEM. *: P<0.05 vs. respective vehicle group. D: Histopathological scores in the different experimental groups. Each symbol represents an individual animal; the horizontal lines with errors represent the mean±SEM. St: single treatment; Rt: 5-day repeated treatment. IMQ: imiquimod.

LOCAL MARKERS OF INFLAMMATION

Overall, both imiquimod and LPS induced a treatment duration-related up-regulation of inflammatory markers (Fig. 2).

No expression changes were observed after a single dose of imiquimod; however, a moderate up-regulation of the pro-inflammatory cytokines IL-6 and IL-1240 was observed after the 5-day repeated treatment. LPS resulted in an up-regulation of all pro-inflammatory cytokines tested after a single administration and a further enhancement with the repeated treatment. The

highest response was observed for IFN α1, which showed more than a 1000-fold up-regulation after the repeated treatment with LPS.

The anti-inflammatory cytokine IL-10 was detected in all groups. While imiquimod showed a tendency to down-regulate IL-10, LPS caused a significant treatment duration-related up-regulation (Fig. 2).

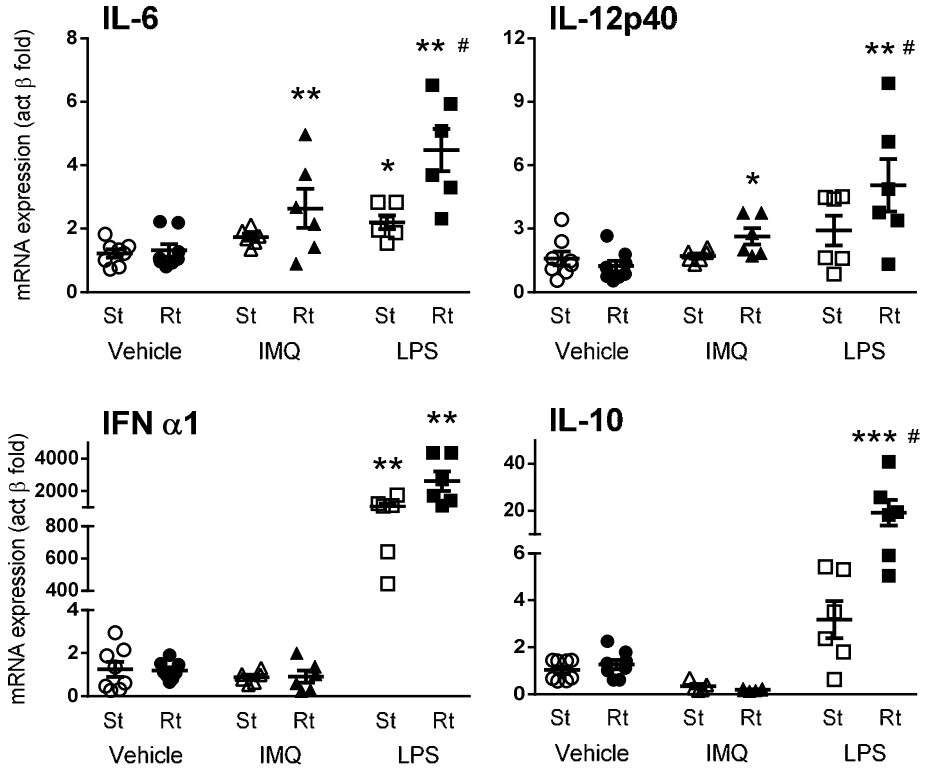


Figure 2. Colonic expression of inflammatory markers. Each symbol represents an individual animal; the horizontal lines with errors represent the mean±SEM. *, **, ***: P<0.05, P<0.01 or P<0.001 vs. corresponding vehicle group. #: P<0.05 vs. single treatment with LPS. St: single treatment; Rt: 5-day repeated treatment. IMQ: imiquimod.

CHARACTERIZATION OF LUMINAL AND WALL-ADHERED MICROBIOTA

Total luminal microbial counts ranged between 4.25 10^{10} cells/ml to 5.51 10^{10} cell/ml, as quantified by DAPI staining. Similar counts were found when assessing total bacteria (EUB338;

3.21 10^{10} cells/ml to 4.78 10^{10}cells/ml); with no differences across experimental groups. HPMC, which can be a substrate for bacterial fermentation, did not modify the luminal microbiota (Table 3). Relative composition of the luminal microbiota was relatively constant, regardless the treatment considered. Gram positive bacteria (*Clostridium coccoides – Eubacterium rectale*>Bifidobacteria >*Lactobacillus - Enterococcus*) represented the vast majority (by 99%). Gram negative groups were much less abundant, with Enterobacteria and Verrucobacteria being very scarce (Table 2; Fig. 3).

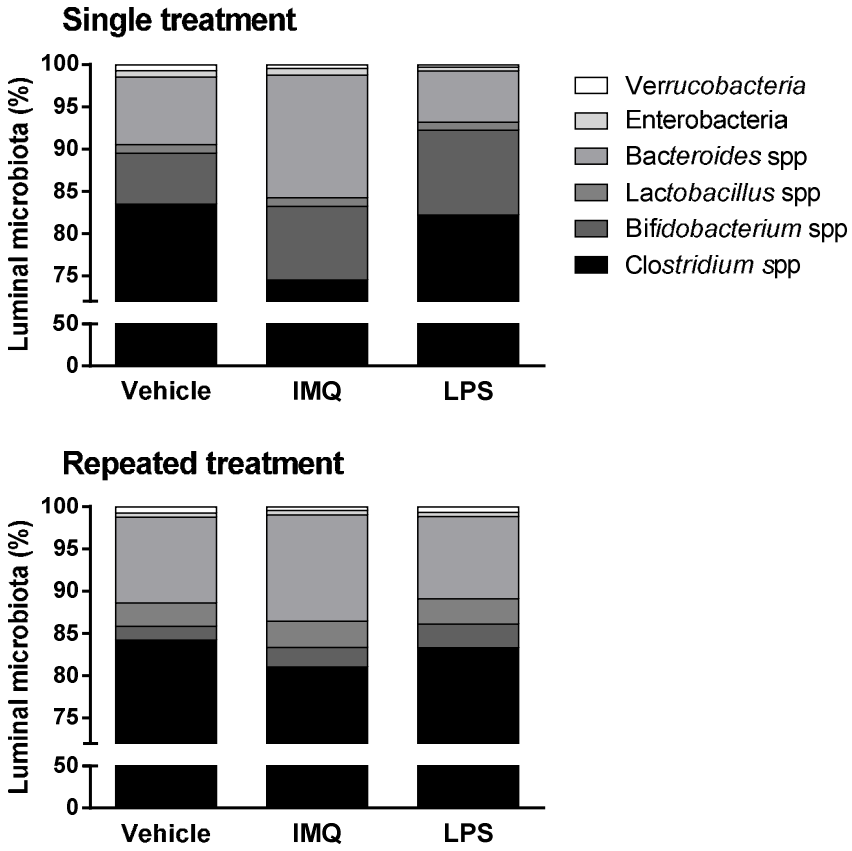


Figure 3. Relative distribution of the luminal colonic microbiota. Data represent the relative abundance (percent) of the main bacterial groups present in the colonic microbiota as quantified using FISH. Relative percent composition was calculated taking as 100% the total counts of the different bacterial groups assessed (as indicate in the legend). For exact bacterial counts, see Table 2.

Table 2. Composition of the colonic luminal commensal microbiota (x10^8 cells/ml).

	DAPI	Total bacteria	*Bacteroides* spp.	Entero-bacteria	Verruco-bacteria	*Clostridium coccoides* cluster XIVa	*Lactobacillus-Enterococcus* spp.	*Bifidobacterium* spp
	Single treatment							
Saline	461±12.8	321±30.7	20.4±4.18	0.14±0.06	1.68±0.08	175±9.51	2.64±0.02	18.6±9.2
HPMC	425±13	353±22.8	13.7±2.3	0.16±0.07	0.76±0.01	162±13.2	1.31±0.3	6.31±2
IMQ	442±20.2	345±31.3	33.2±6.4	0.22±0.04	1.23±0.7	136±11.4	1.3±0.4	20.2±9.23
LPS	461±15.4	350±25.8	12.3±4.39	0.09±0.04	0.22±±0.07	145±24.3	1.4±0.3	22.2±4.91

	DAPI	Total bacteria	*Bacte-roides* spp.	Entero-bacteria	Verruco-bacteria	*Clostridium coccoides* cluster XIVa	*Lacto-bacillus Entero-coccus* spp.	*Bifidobacterium* spp
	Repeated treatment							
Saline	500±28.8	378±53.7	21.3±9.25	0.06±0.03	2.35±0.7	179±21.6	4.21±1.28	1.4±0.8
HPMC	544±21.1	430±11.9	25.7±57.6	0.09±0.07	1.07±0.2	212±21.8	8.59±3.69	6.14±2.22
IMQ	551±40.8	478±99.9	28.5±14	0.25±0.1	1.08±0.29	183±18.3	7.22±2.21	5.32±1.61
LPS	540±27.2	449±40.8	20.4±5.8	0.21±0.08	1.66±0.8	175±12.2	6.33±1.95	6.17±2.08

[a]: Data are mean±SEM, n=4-6 per group. (ANOVA).

In single or repeated vehicle-treated animals, the most abundant bacteria adhered to the colonic wall was *Clostridium* spp (with an incidence of attachment from 50% to 90%) followed by *Bifidobacterium* spp. (incidence of attachment: 10% to 50%). Imiquimod, single or repeated treatment, did not affect the rates of bacterial adherence. Conversely, treatment with LPS favored bacterial adherence, increasing the rates of Clostridia and Bifidobacteria attachment up to 100% and 70%, respectively; however, statistical significance was not reached (Fig. 4). Other bacterial groups were only occasionally observed attached to the colonic epithelium, with no consistent treatment-related pattern.

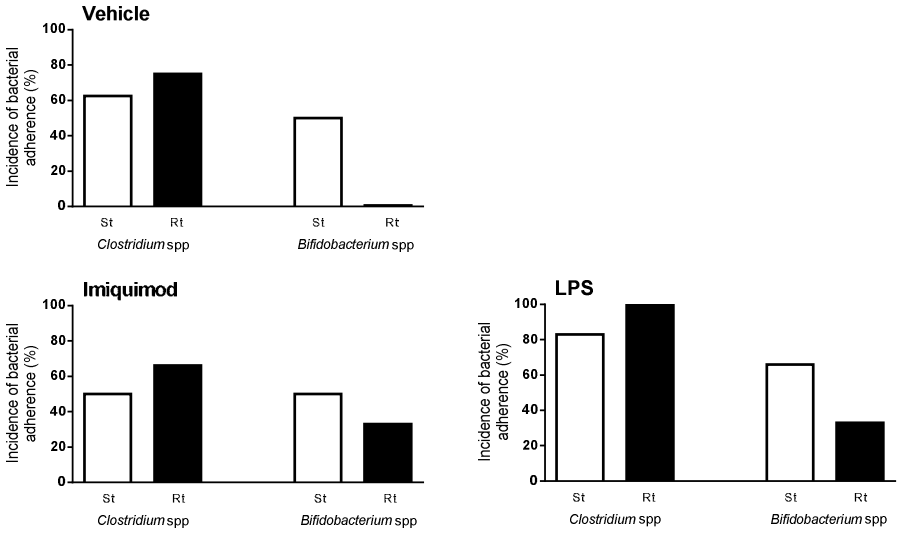


Figure 4. Incidence of *Clostridium* spp. and *Bifidobacterium* spp. attachment to the colonic epithelium. Data represent the percentage of animals showing bacteria attached to the epithelium. St: single treatment; Rt: 5-day repeated treatment.

S-IGA LEVELS AND S-IGA COATED BACTERIA

S-IgAs were detected in all luminal samples analyzed. Only the repeated treatment with LPS resulted in a significant variation in luminal s-IgA levels, with a 45% reduction ($P<0.05$; Table 3).

IgA-coated bacteria were observed in all luminal samples analyzed. Only the repeated treatment with LPS resulted in a significant variation in the counts of luminal IgA-coated bacteria, with a 59% increase compared with vehicle-treated animals ($P<0.05$; Table 3).

Table 3. Luminal s-IgA and IgA-coated bacteria. [a]

	Luminal s-IgA (mg/dL)		**IgA-coated bacteria (x10^8 cells/ml)**	
	Single treatment	*Repeated treatment*	*Single treatment*	*Repeated treatment*
Vehicle	55.45±3.59	25.14±3.14	22.54±9.57	22.20±1.16
Imiquimod	54.23±9.6	29.03±7.4	27.01±9.16	33.7±5.03*
LPS	40.96±7.96	18.32±2.15 *	15.75±2.62	29.22±2.40*

[a]:Data are mean±SEM of n=6-8 per group. *: $P<0.05$ vs. respective control (ANOVA).

COLONIC EXPRESSION OF HOST-BACTERIAL INTERACTION MARKERS: TLRS AND INTEGRINS

TLRs expression was detected in all colonic samples analyzed. In control conditions, relative expression of TLRs was TLR4> TLR5> TLR2> TLR7. The 5-day repeated treatment with imiquimod resulted in an up-regulation of TLR4 (by 41%; P<0.05) and TLR7 (by 94%; P<0.05; Fig. 5). LPS had a more extended effect, up-regulating TLRs expression after both a single or repeated administration (Fig. 5).

Expression of integrins (Itg) α2 and β1, was slightly, but not-significantly (by 0.5-fold) increased by imiquimod. However, LPS, after either a single or repeated administration, resulted in a similar up-regulation of both Itgs (Fig. 5).

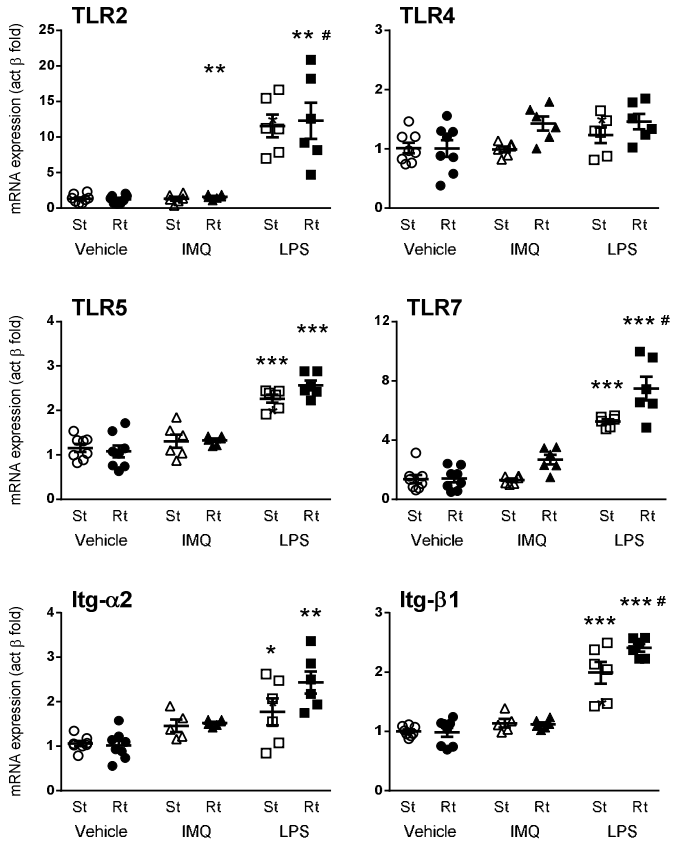


Figure 5. Colonic expression of host-bacterial interaction markers: TLRs and integrins α2 (Itg-α2) and β1 (Itg-β1). Each symbol represents an individual animal; the horizontal lines with errors represent the mean±SEM. *, **, ***: P<0.05, P<0.01 or P<0.001 vs. corresponding vehicle group. #: P<0.05 vs. single treatment with LPS. St: single treatment; Rt: 5-day repeated treatment. IMQ: imiquimod.

COLONIC EXPRESSION OF ANTIMICROBIAL PEPTIDES

From all antimicrobial peptides tested, defensin β4 was not detectable in any of the samples analyzed. In control conditions, RELMβ showed the highest expression levels, while defensin α24 and Reg3γwere almost undetectable. Imiquimod, single or repeated treatment, had no significant effects on the expression of antimicrobial peptides (Fig. 6). On the other hand, LPS, either after a single or repeated administration, up-regulated defensin α24, Reg3γ and RELMβ, although a relatively high interindividual variability was observed in some cases (Fig. 6).

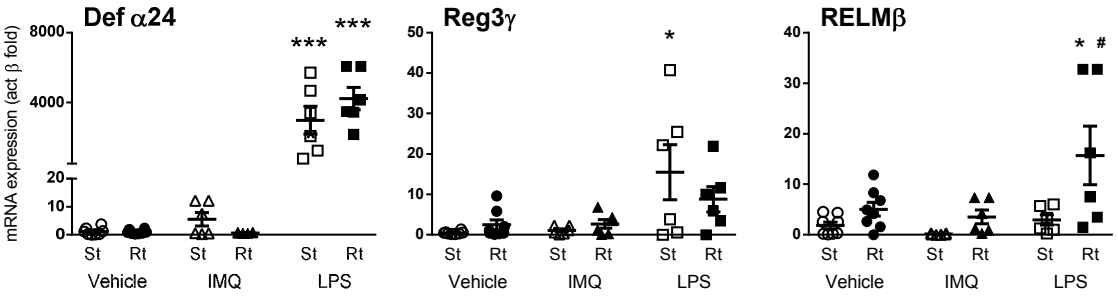


Figure 6. Colonic expression of antimicrobial peptides: defensin α24 (Def α24), regenerating islet-derived protein 3 gamma (REg3γ) and resistin-like molecule beta (RELMβ). Each symbol represents an individual animal; the horizontal lines with errors represent the mean±SEM. *, ***: P<0.05 or P<0.001 vs. corresponding vehicle group. #: P<0.05 vs. single treatment with LPS. St: single treatment; Rt: 5-day repeated treatment. IMQ: imiquimod.

COLONIC EXPRESSION OF SENSORY-RELATED MARKERS

Expression of all sensory-related markers assessed was detected in colonic samples. In control conditions, relative expression levels were PAR-2 > FAAH > TRPV3 > TRPV4 > TRPV1 > CB1 > CB2 > MOR > CGRP. Imiquimod had marginal effects on sensory-related markers, with only a tendency to down-regulate TRPV1 and TRPV3 and to up-regulate CB2 expression (Fig. 7). However, LPS, after single or repeated administration, resulted in a consistent up-regulation of cannabinoid receptors, TRPV1/3/4 and CGRP (Fig. 7).

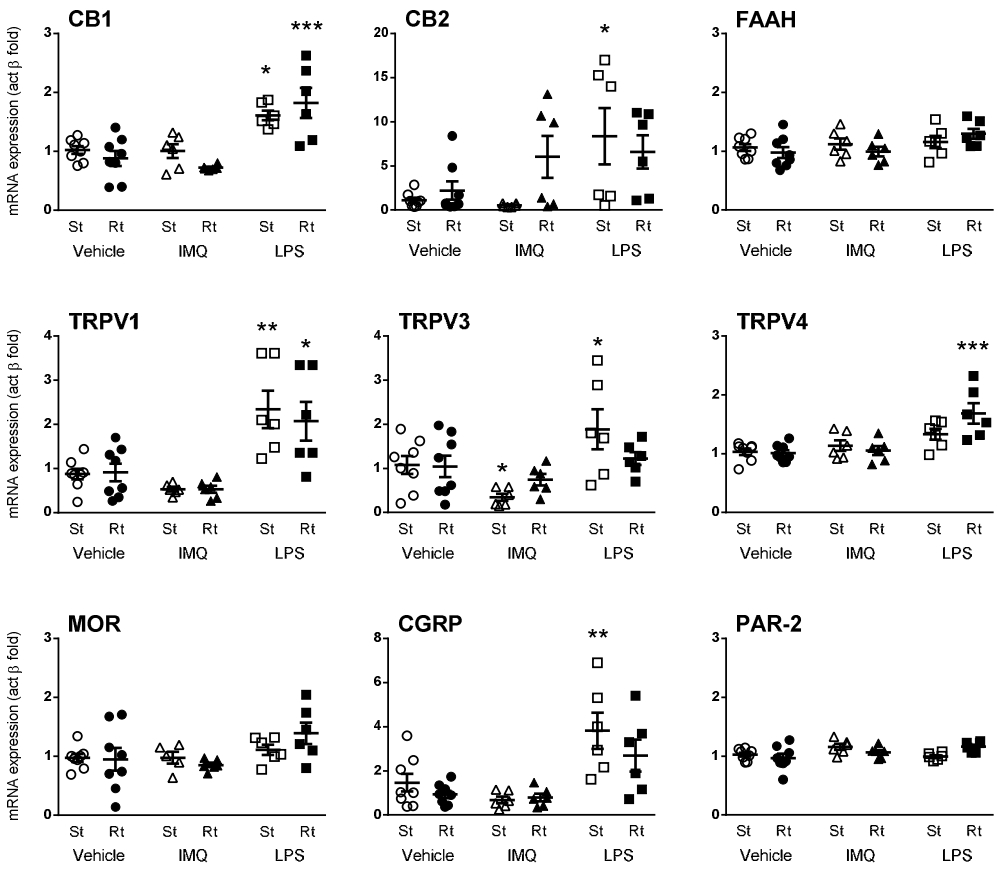


Figure 7. Colonic expression of sensory-related markers: Cannabinoid receptors 1 (CB1) and 2 (CB2), fatty acid amide hydrolase (FAAH), transient receptor potential vanilloid 1 (TRPV1), 3 (TRPV3) and 4 (TRPV4), μ opioid receptor (MOR), calcitonin gene-related peptide (CGRP) and proteinase activated receptor 2 (PAR-2). Each symbol represents an individual animal; the horizontal lines with errors represent the mean±SEM. *, ***: P<0.05 or P<0.001 vs. corresponding vehicle group. #: P<0.05 vs. single treatment with LPS. St: single treatment; Rt: 5-day repeated treatment. IMQ: imiquimod.

DISCUSSION

Changes in the composition of the commensal microbiota with alterations in host-microbial interactions and/or in local neuro-immune responses have been suggested as key factors for the development in inflammatory and functional alterations within the gastrointestinal tract. In this study, we directly stimulated components of the innate immune system related to bacterial recognition (namely TLR4 and 7) in order to characterize changes in neuro-immune mechanisms within the gut elicited by the interaction with microbial components. We found that the direct stimulation of TLR4 and 7 leads to a time-related, TLR-specific local activation of the

immune system and a modulation of host-bacterial interaction and sensory-related systems, with minor changes in the commensal microbiota, per se.

Within the gut, TLR4 and TLR7 have been implicated in the genesis of host responses to the microbiota. To simulate a state of altered microbiota, leading to an over-stimulation of these receptors, we administered locally selective agonists, LPS and imiquimod, which are likely to activate TLR4- or TLR7-dependent signaling cascades, respectively, in a selective manner. Indeed, we observed a local immune activation, as indicated by the up-regulation of pro- and anti-inflammatory cytokines. These changes occurred, however, in the absence of structural (macro or microscopical) alterations consistent with the induction of inflammation. This is coherent with previous reports in which similar procedures lead to an immune activation in the absence of overt inflammation. [30–32] The only structural alteration observed was a transitory change in goblet cells, with higher ratios of mixed mucins, after a single treatment with LPS. [33] However, this seems to be quickly compensated by the host, as no similar alterations were found after repeated exposure, in agreement with that previously reported. [32]A reason for the lack of clear inflammation could be the duration of the treatment. Since immune responses were enhanced with the repeated treatment, it is feasible to speculate that longer-lasting treatments (simulating a more sustained state of dysbiosis) could lead to an overt state of colitis. Overall, LPS was more effective than imiquimod up-regulating pro-inflammatory cytokines. This agrees with the described effect of bacterial LPS altering gut homeostasis and leading to systemic responses after local administration. [32] Interestingly, and particularly for LPS, the up-regulation of pro-inflammatory cytokines coincided with an up-regulation of IL-10, the main anti-inflammatory cytokine. This indicates the onset of compensatory mechanisms to a pro-inflammatory state and might explain the absence of an over inflammatory response.

Activation of TLR4 and 7 was also associated with changes in host-bacterial interaction systems, including the self-regulation of TLRs. Again, as mentioned above, responses were more evident for the LPS-mediated stimulation of TLR4 and enhanced during the repeated treatment. Alterations in TLR4 signaling have been linked to changes in IgA levels. Here, in particular, we observed diminished levels of free luminal secretory-IgA during the repeated treatment with LPS, thus agreeing with data showing that systemic LPS decreased intestinal s-IgA levels. [34] However, an increase in the counts of luminal IgA-coated bacteria were observed during repeated treatment with either LPS or imiquimod. The relationship between free s-IgA and IgA-coated bacteria is not

clear. Numerous environmental factors (diet, microbial components,…) and inflammation per se can modify the number of IgA-coated bacteria. [29,35] It is known that commensal bacteria stimulates the production and secretion of s-IgA and that s-IgA-coated bacteria are unable to adhere to or penetrate the mucosal barrier and are efficiently eliminated from the host. [36] Therefore, an increase in the ratio of s-IgA-coated bacteria might represent a defensive response of the host in a state of dysbiosis and might suggest an increased secretory activity, although the low levels of free luminal s-IgA detected. This coincided also with an increase in the expression of antimicrobial peptides, seen particularly during repeated LPS treatment. This is consistent with previous studies which described that bacterial ligands, including LPS, and direct TLR activation are sufficient to trigger the expression of antimicrobial peptides. [2,37,38] All together, these changes might reflect and active response of the host towards the increased TLR-mediated signaling, which might be interpreted as a dysbiotic state with the need of the activation of microbiota-controlling mechanisms.

These changes are also coherent with the presence of alterations in host-bacterial interaction systems. Sings of dysbiosis, indicated by the increased TLR-mediated signaling, modulated the expression of molecules related to epithelium-bacteria interactions that can act as bacterial receptors, such as integrins, facilitating bacterial aggregation and attachment. [39,40] Integrins, particularly β2, can also act as modulators or TLR-mediated signaling, avoiding exaggerated responses due to excessive TLR stimulation. [41] Overall, the changes described suggest the generation of a host response directed towards the reshaping of the microbiota, the control of the immune response and, although not assessed here, the prevention of bacterial translocation. In agreement, work in animal models of colitis has shown that LPS and imiquimod ameliorate colitis by enhancing the expression of cytokines and antimicrobial peptides. [31,42,43]

Although the host responses described above, luminal microbiota was not affected during TLR4/7 stimulation. However, repeated treatment with LPS seemed to favor bacterial (Clostridia and Bifidobacteria) attachment to the colonic wall. This is consistent with the expression changes in TLRs and integrins, as described above, which might act as factors modulating (favoring) bacterial attachment. Bacterial adherence to the intestinal epithelium is important because adhered microbiota is the one directly interacting with the host's bacterial recognition systems and, therefore, generating beneficial or harmful responses within the gut.[44,45] Increased bacterial adherence seem to be important to maintain mucosal inflammation and is commonly observed in

states of immune activation, such as during intestinal inflammation or in functional gastrointestinal diseases. [23,46,47]

In addition to an immune modulation, stimulation of TLR-dependent signaling cascades lead also to a modulation of sensory-related systems within the colon. The existence of a crosstalk between the immune and the enteric nervous system is widely accepted. Together with previous observations, our data clearly supports the existence of a functional axis connecting the microbiota, the immune systems and the enteric nervous system. [5,11,12] Our results show that the selective stimulation of TLR4, but not TLR7, resulted in an up-regulation of several sensory-related markers directly implicated in viscerosensitivity. Recently, a potential role for TLR4 directly modulating visceral sensitivity has been proposed, suggesting that TLR4-dependent cytokines production might be necessary for the development of stress-induced visceral hypersensitivity.[48–51] Results obtained here might represent an insight into the pathways leading to TLR4-mediated altered visceral sensitivity, which might implicate modulation of cannabinoid and TRPV receptors and CGRP content. In addition, these observations further support a direct action of microbiota regulating sensory systems within the gut, as observed in states of dysbiosis, during spontaneous adaptive changes of the microbiota or during the administration of certain probiotic strains. [11–14] These observations warrant follow up studies, outside the original scope of the present work, assessing the potential functional consequences of the changes observed here in sensory markers. A functional translation of the present molecular observations is supported, for instance, by data indicating that LPS activates visceral afferents and can generate states of visceral hyperalgesia both in animals and humans. [48–52]

In summary, we found that simulation of a dysbiotic state with altered microbial-derived signaling to the host by the selective stimulation of colonic TLR4 and 7 induces a local immune activation, alters host-bacterial interactions and leads a modulation of sensory-related systems. Responses observed were TLR- and time-dependent, with enhanced responses associated to the repetitive LPS-mediated stimulation of TLR4; thus suggesting that prolonged changes in mainly LPS-producing Gram negative bacteria (signaling through TLR4) might have the major impact in gut homeostasis. Overall, changes observed might represent a part of the host's response to an alteration of the microbiome, with the objective of regaining a balance within the commensal microbiota, avoiding excessive immune responses and the development of active (structural) inflammation.

ACKNOWLEDGMENTS

Martínez and A. Acosta are thanked for their technical assistance. This study was supported by grants BFU2009-08229 and BES-2010-037699 (FPI program; M.A. personal support) from the Ministerio de Ciencia e Innovación (Spain) and 2009SGR708 from the Generalitat de Catalunya.

The authors do not have any conflict of interest.

REFERENCES

1. Garrett WS, Gordon JI, Glimcher LH. Homeostasis and inflammation in the intestine. Cell 2010;140:859–870.

2. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science 2011;334:255–258.

3. Hooper L V, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol 2010;10:159–169.

4. Koboziev I, Reinoso Webb C, Furr KL, Grisham MB. Role of the enteric microbiota in intestinal homeostasis and inflammation. Free Radic Biol Med 2014;68:122–133.

5. Costes LM, Boeckxstaens GE, de Jonge WJ, Cailotto C. Neural networks in intestinal immunoregulation. Organogenesis 2013;9:216–223.

6. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 2012;10:735–742.

7. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol 2013;14:685–690.

8. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 2008;8:411–420.

9. Lavelle EC, Murphy C, O'Neill LAJ, Creagh EM. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. Mucosal Immunol 2010;3:17–28.

10. Kubinak JL, Round JL. Toll-like receptors promote mutually beneficial commensal-host interactions. PLoS Pathog 2012;8:e1002785.

11. Aguilera M, Vergara P, Martínez V. Environment-related adaptive changes of gut commensal microbiota do not alter colonic toll-like receptors but modulate the local expression of sensory-related systems in rats. Microb Ecol 2013;66:232–243.

12. Aguilera M, Vergara P, Martínez V. Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. Neurogastroenterol Motil 2013;25:e515–529.

13. Verdú EF, Bercik P, Verma-Gandhu M, Huang X-X, Blennerhassett P, Jackson W, et al. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 2006;55:182–190.

14. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 2007;13:35–37.

15. Distrutti E, Cipriani S, Mencarelli A, Renga B, Fiorucci S. Probiotics VSL#3 protect against development of visceral pain in murine model of irritable bowel syndrome. PLoS One 2013;8:e63893.

16. Agostini S, Goubern M, Tondereau V, Salvador-Cartier C, Bezirard V, Lévèque M, et al. A marketed fermented dairy product containing Bifidobacterium lactis CNCM I-2494 suppresses gut hypersensitivity and colonic barrier disruption induced by acute stress in rats. Neurogastroenterol Motil 2012;24:376–e172.

17. Johnson AC, Greenwood-Van Meerveld B, McRorie J. Effects of Bifidobacterium infantis 35624 on post-inflammatory visceral hypersensitivity in the rat. Dig Dis Sci 2011;56:3179–3186.

18. Kamdar K, Nguyen V, DePaolo RW. Toll-like receptor signaling and regulation of intestinal immunity. Virulence 2013;4:207–212.

19. Gómez-Llorente C, Muñoz S, Gil A. Role of Toll-like receptors in the development of immunotolerance mediated by probiotics. Proc Nutr Soc 2010;69:381–389.

20. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 2010;10:131–144.

21. Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, et al. Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. J Histochem Cytochem 2009;57:1013–1023.

22. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000;68:7010–7017.

23. Terán-Ventura E, Aguilera M, Vergara P, Martínez V. Specific changes of gut commensal microbiota and TLRs during indomethacin-induced acute intestinal inflammation in rats. J Crohns Colitis 2014; 8:1043-1054.

24. Igarashi H, Ohno K, Maeda S, Kanemoto H, Fukushima K, Uchida K, et al. Expression profiling of pattern recognition receptors and selected cytokines in miniature dachshunds with inflammatory colorectal polyps. Vet Immunol Immunopathol 2014;159:1–10.

25. Brint EK, MacSharry J, Fanning A, Shanahan F, Quigley EMM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. Am J Gastroenterol 2011;106:329–336.

26. Melgar S, Engström K, Jägervall A, Martinez V. Psychological stress reactivates dextran sulfate sodium-induced chronic colitis in mice. Stress 2008;11:348–362.

27. Selinummi J, Seppälä J, Yli-Harja O, Puhakka JA. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 2005;39:859–63.

28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408.

29. Massot-Cladera M, Pérez-Berezo T, Franch A, Castell M, Pérez-Cano FJ. Cocoa modulatory effect on rat faecal microbiota and colonic crosstalk. Arch Biochem Biophys 2012;527:105–112.

30. Martinez V, Melgar S. Lack of colonic-inflammation-induced acute visceral hypersensitivity to colorectal distension in Na(v)1.9 knockout mice. Eur J Pain 2008;12:934–944.

31. Im JP, Ye BD, Kim JM, Jung HC, Song IS, Kim JS. Rectal administration of lipopolysaccharide and ovalbumin ameliorates acute murine colitis. Dig Dis Sci 2011;56:2292–2298.

32. Im E, Riegler FM, Pothoulakis C, Rhee SH. Elevated lipopolysaccharide in the colon evokes intestinal inflammation, aggravated in immune modulator-impaired mice. Am J Physiol Gastrointest Liver Physiol 2012;303:G490–497.

33. Chen L, Wang T, Zhang J-Y, Zhang S-F, Liu D-S, Xu D, et al. Toll-like receptor 4 relates to lipopolysaccharide-induced mucus hypersecretion in rat airway. Arch Med Res 2009;40:10–17.

34. Liu C. Changes in intestinal mucosal immune barrier in rats with endotoxemia. World J Gastroenterol 2009;15:5843.

35. Van der Waaij LA, Kroese FGM, Visser A, Nelis GF, Westerveld BD, Jansen PLM, et al. Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. Eur J Gastroenterol Hepatol 2004;16:669–674.

36. Macpherson AJ, McCoy KD, Johansen F-E, Brandtzaeg P. The immune geography of IgA induction and function. Mucosal Immunol 2008;1:11–22.

37. Brandl K, Plitas G, Schnabl B, DeMatteo RP, Pamer EG. MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J Exp Med 2007;204:1891–1900.

38. Kinnebrew MA, Ubeda C, Zenewicz LA, Smith N, Flavell RA, Pamer EG. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. J Infect Dis 2010;201:534–543.

39. Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Bronson RT, et al. Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. Proc Natl Acad Sci U S A 2007;104:15823–15828.

40. Sinclair JF, Dean-nystrom EA, Brien ADO. The Established Intimin Receptor Tir and the Putative Eucaryotic Intimin Receptors Nucleolin and ␣L 1 Integrin Localize at or near the Site of Enterohemorrhagic Escherichia coli O157 : H7 Adherence to Enterocytes In Vivo 2006;74:1255–1265.

41. Yee NK, Hamerman J a. β(2) integrins inhibit TLR responses by regulating NF-κB pathway and p38 MAPK activation. Eur J Immunol 2013;43:779–792.

42. Sainathan SK, Bishnupuri KS, Aden K, Luo Q, Houchen CW, Anant S, et al. Toll-like receptor-7 ligand Imiquimod induces type I interferon and antimicrobial peptides to ameliorate dextran sodium sulfate-induced acute colitis. Inflamm Bowel Dis 2012;18:955–967.

43. Saito K, Katakura K, Suzuki R, Suzuki T, Ohira H. Modulating Toll-like receptor 4 signaling pathway protects mice from experimental colitis. Fukushima J Med Sci 2013;59:81–88.

44. Lu L, Walker WA. Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. Am J Clin Nutr 2001;73:1124S–1130.

45. Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans ADL, de Vos WM. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol 2002;68:3401–3407.

46. Swidsinski A, Loening-Baucke V, Lochs H, Hale L-P. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World J Gastroenterol 2005;11:1131–1140.

47. Carroll IM, Ringel-Kulka T, Keku TO, Chang Y-H, Packey CD, Sartor RB, et al. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2011;301:G799–807.

48. Tramullas M, Finger BC, Moloney RD, Golubeva A V, Moloney G, Dinan TG, et al. Toll-like receptor 4 regulates chronic stress-induced visceral pain in mice. Biol Psychiatry 2014;76:340–348.

49. Meseguer V, Alpizar YA, Luis E, Tajada S, Denlinger B, Fajardo O, et al. TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. Nat Commun 2014;5:3125.

50. Sauer R-S, Hackel D, Morschel L, Sahlbach H, Wang Y, Mousa SA, et al. Toll like receptor (TLR)-4 as a regulator of peripheral endogenous opioid-mediated analgesia in inflammation. Mol Pain 2014;10:10.

51. Chen Z-Y, Zhang X-W, Yu L, Hua R, Zhao X-P, Qin X, et al. Spinal toll-like receptor 4-mediated signalling pathway contributes to visceral hypersensitivity induced by neonatal colonic irritation in rats. Eur J Pain 2014; doi: 10.1002/ejp.534..

52. Benson S, Kattoor J, Wegner A, Hammes F, Reidick D, Grigoleit J-S, et al. Acute experimental endotoxemia induces visceral hypersensitivity and altered pain evaluation in healthy humans. Pain 2012;153:794–799.

CHAPTER 3

STRESS AND ANTIBIOTICS ALTER LUMINAL AND WALL-ADHERED MICROBIOTA AND ENHANCE THE LOCAL EXPRESSION OF VISCERAL SENSORY-RELATED SYSTEMS IN MICE

M. Aguilera[a,c], P. Vergara[a,b,c], V. Martínez[a,b,c]

[a]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain.

[b]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Spain.

[c]Neuroscience Institute, Universitat Autònoma de Barcelona, Barcelona, Spain.

ABSTRACT

Stress leads to altered gastrointestinal neuro-immune responses. We characterized the interaction between stress and gut commensal microbiota and their role modulating colonic responses to stress, the induction of inflammation, the expression of sensory-related markers and visceral sensitivity. C57BL/6 female mice were treated (7 days, PO) with non-absorbable-broad spectrum antibiotics (bacitracin/neomycin, 0.4 mg/mouse/day). Simultaneously, mice were subjected to a 1 h/day (7 days) session of psychological stress (water avoidance stress, WAS). Luminal and wall-adhered microbiota were characterized by fluorescent *in situ* hybridization. Cannabinoid receptors 1 and 2 (CB1/2), tryptophan hydroxylase 1 and 2 (TPH1/2) and inflammatory markers were quantified by RT-qPCR and Secretory-IgA (s-IgA) by ELISA. Visceral sensitivity was assessed after the intracolonic administration of capsaicin. Antibiotics did not affect the defecatory and endocrine responses to stress. However, antibiotics diminished by 2.5-folds total bacterial counts, induced a specific dysbiosis and favored bacterial wall adherence. Combining antibiotics and stress resulted in further reductions in bacterial counts and a dysbiosis, with enhanced bacterial wall adherence. Luminal s-IgA levels were enhanced in dysbiotic mice. Nevertheless, no alterations consistent with the induction of colonic inflammation were observed. Dysbiosis up-regualted CB2 expression and WAS up-regulated both CB2 and TPH1 expression. In dysbiotic mice, visceral pain-related responses were reduced. Manipulations of the commensal microbiota and the interaction host-microbiota are able to modulate the local expression of neuro-immune-endocrine systems within the colon, leading to a modulation of visceral sensitivity. These mechanisms might contribute to the pathogenic and protective roles of microbiota in gastrointestinal homeostasis.

Key words: endocannabinoid system; gut commensal microbiota; intestinal dysbiosis; secretory-IgA; serotonergic system; visceral pain

Chapter 3

INTRODUCTION

Functional gastrointestinal disorders, represented mainly by irritable bowel syndrome (IBS), are among the most prevalent gastrointestinal alterations in the western population. Alterations in bowel habits, abdominal pain and discomfort, believed to reflect increased visceral sensitivity, are hallmarks of IBS.[1] Symptoms in IBS fluctuate over time in intensity and character, but the mechanisms underlying these cycles are unclear. Several factors, including stress, intestinal infection, drugs and diet have been reported to exacerbate symptomatology, and might be key components of the pathophysiology of the disease.[2,3] A growing body of evidence suggests that IBS pathogenesis is likely dependent on the interaction between local immune reactions within the intestinal wall and environmental factors in genetically susceptible individuals. In particular, stress and perturbations of the gut commensal microbiota have been recognized as two potential factors contributing to the onset, maintenance and exacerbation of both functional and inflammatory gastrointestinal disorders.[4,5] Indeed, stressful life events or depression are risk factors for the onset or relapse of intestinal inflammation and for symptoms presentation in IBS patients. Similarly, growing evidences suggest that IBS patients have a dysbiotic intestinal microbiota.[4,6] Despite these evidences, the exact role of gut microbiota and stress, individually or as interactive factors, in the pathophysiology of IBS remains largely unknown.

In this study, we characterized the interaction between stress and microbiota and their potential role modulating functional colonic responses to stress and the induction of inflammatory-like changes in mice. First, we assessed the effects of repetitive psychological stress (water avoidance stress, WAS) and antibiotic treatment, individually or in combination, on the composition of ceco-colonic commensal microbiota and the induction of inflammatory-like changes in the colon. In the same animals, endocrine and colonic motor responses to stress were assessed simultaneously. To characterize the ceco-colonic microbiota we determined changes in both luminal and wall (epithelium)-adhered microbiota. The assessment of inflammatory responses was based on inflammatory markers, histological evaluation of the colon and quantification of luminal secretory-IgA (s-IgA). s-IgA is considered the main anti-inflammatory immunoglobulin of the mucosal intestinal immune system regulating the number, composition and functions of luminal bacteria.[7,8] Moreover, we also determined changes in relevant systems that have been involved in sensory responses within the colon, with particular relevance to IBS, namely the endocannabinoid and the serotonergic systems. For this, colonic expression of

cannabinoid receptors type 1 and 2 (CB1 and CB2) and activity of the serotonergic system [density of enterochromaffin cells and expression of the tryptophan hydroxylase isoform 1 and 2 (TPH1 and TPH2)] were characterized in the same animals. Finally, to determine if these alterations translate into functional changes in visceral sensitivity we tested visceral pain-related responses in animals treated with antibiotics, with or without the addition of stress. For this, we assessed the presence of visceral pain-related behaviours associated to the intracolonic administration of capsaicin, as previously described.[9,10]

MATERIALS AND METHODS

ANIMALS

Female C57Bl/6 mice, 6 week-old (Charles River Laboratories, Lyon, France) were used. Upon arrival, animals were acclimatized for a 1-week period prior to any experimentation. All animals were maintained in standard conditions in an environmentally controlled room (20-22ºC, 12 h light:dark cycle), with food and water *ad libitum*. 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 TREATMENT

Animals received a mixture of non-absorbable, broad spectrum antibiotics containing Bacitracin A (Vetranal™; Sigma-Aldrich, Barcelona, Spain) and Neomycin (Neomycin trisulfate salt hydrate; Sigma-Aldrich). Amphotericin B (Sigma-Aldrich) was added to prevent yeast overgrowth. Animals were dosed by oral gavage with 0.3 mL of the antibiotic/antifungal mixture, during 7 consecutive days. In addition, the same antibiotic/antifungal mixture was added to the drinking water during the same period of time. This protocol ensured a minimum dose of 0.4 mg for bacitracin and neomycin and 0.1 mg for amphotericin B (per mouse and day). Vehicle-treated animals received vehicle (deionised water) by oral gavage (0.3 mL) and normal drinking water during the same period of time. Water consumption, assessed on a daily basis during the treatment period, was similar across groups (data not shown). Similar treatment protocols have

been followed previously in comparable studies in mice and rats, demonstrating the induction of significant changes of the commensal microbiota.[11-13]

Repetitive psychological stress (Water avoidance stress)

Water avoidance stress (WAS) was performed following previous protocols described by us.[14,15] Animals were placed on a platform (4-cm diameter, 6-cm height) located in the center of a standard plastic cage (530x280x155 mm) filled with tap water (18–20 ºC) to about 1 cm below the edge of the platform. Stress sessions lasted for 1h and were repeated on 7 consecutive days. Control animals were maintained in their home cages. All procedures were performed in the morning (finishing no later than 12:00 h) to minimize any influence of circadian rhythms. Fecal pellet output during the 1-h session of WAS/non-stress was used as a marker of stress.

Behavioral responses to intracolonic capsaicin-evoked visceral pain

Spontaneous visceral pain-related behaviours induced by intracolonic capsaicin were assessed following previously described protocols, with minor modifications.[9,10] Mice were anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and capsaicin (0.05 ml/mice, 0.1% in ethanol:Tween 80:saline; 1:1:8, v:v:v; Sigma-Aldrich) was administered intracolonically (about 4 cm from the anus) with a rounded tip plastic cannula (length 7.5 cm, diameter 0.61mm). Petroleum jelly was applied on the perianal area to avoid stimulation of somatic areas through contact with capsaicin. Animals were placed in plastic cages (20x20x14 cm) and, after recovering consciousness, visceral pain-related behaviours were assessed during a 30 min period. Pain behaviours were visually assessed by two independent researchers (MA and VM). Behaviours assessed included: licking of the abdomen, stretching of abdomen, squashing the abdomen to the floor, and abdominal retractions. For each animal, the number of behaviours for the 30 min observation time was determined as the mean of the quantification performed by the two observers.

Experimental protocols

Mice (n=24) were randomly divided into 4 experimental groups (n=6 each): i) vehicle-treated non-stressed mice; ii) vehicle-treated stressed mice; iii) antibiotic-treated non-stressed mice; and iv) antibiotic-treated stressed mice. Animals were treated with antibiotics or vehicle for

a period of 7 days, as described above. In addition, from day 2 to 8 animals were subjected to a 1 h/day session of psychological stress (WAS) or maintained in their home cages (control). On day 8, immediately after the last session of stress, animals were euthanized and blood, tissue (ceco-colonic region) and fecal samples obtained.

In a second experiment, mice (n=20) were divided in the same experimental groups and followed the same treatments (n=5 per group). At the end of treatments, visceral pain-related responses to intracolonic capsaicin were assessed as described above. In this case, at the end of the procedure, animals were euthanized and weight of body organs was assessed (see samples collection).

SAMPLES COLLECTION

Immediately after the last stress session, mice were deeply anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Thereafter, a medial laparotomy was performed, the ceco-colonic region localized and the cecum and colon dissected. Afterward, ceco-colonic fecal contents and a tissue sample from the proximal colon were collected and frozen immediately in liquid nitrogen. Frozen samples were stored at –80 ºC until analysis. At the same time, tissue samples of the proximal and middle colon (about 1.5 cm each) were collected and fixed overnight in Carnoy fixative (ethanol:chloroform:glacial acetic acid, 6:3:1, v:v:v) or in 4% paraformaldehyde. After an overnight fixing, tissues were paraffin embedded and 5 µm-tick sections obtained. In addition, the adrenal glands, the thymus and the spleen were dissected and weighed. Serum was obtained by centrifugation of blood samples (15 min, 2465 g, 4 ºC) and maintained at -80 ºC until analysis. In animals used to assess visceral sensitivity, at necropsy, only weight of body organs was assessed (cecum, adrenal glands, thymus and spleen).

BACTERIAL IDENTIFICATION BY FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

For fluorescence *in situ* hybridization (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). Probes used were: EUB 338 (5'GCTGCCTCCCGTAGGAGT3') to total Bacteria; NON 338 (5'ACATCCTACGGGAGGC3') to non-bacteria (negative control); BAC 303 (5'CAATGTGGGGGACCTT3') to *Bacteroides* spp.; EREC 482

(5'GCTTCTTAGTCAGGTACCG3') to *Clostridium* Cluster XIVa; LAB 158 (5'GGTATTAGCACCTGTTTCCA3') to *Lactobacillus* spp. and *Enterococcus* spp.; ENT-D (5'TGCTCTCGCGAGGTCGCTTCTCTT3') to Enterobacteria; BIF 164 (5'CATCCGGCATTACCACCC3') to *Bifidobacterium* spp.; and MUC-1437 (5'CCTTGCGGTTGGCTTCAGAT 3') to Verrucobacteria.

Fecal samples of ceco-colonic content were used to characterize luminal commensal microbiota. *In situ* hybridization of bacteria in the luminal content was performed on glass slides, as previously described by us.[16,17] Samples were hybridized in a dark moist chamber (for 3 h) by addition of 100 µl hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS at pH 7.2) with the corresponding Cy3-labeled oligonucleotide probe (concentration 5 ng μl^{-1}). Treatments with formamide or lysozyme and hybridization temperatures were used as described to achieve the optimal stringency. After hybridization, the slides were rinsed in a pre-warmed washing buffer (20 mM Tris-HCl, 0.9 M NaCl at pH 7.2) for 30 min and then cleaned with Milli-Q water to remove unbound probes. Washed slides were air-dried and mounted with Vectashield-DAPI (Vector Laboratories, Orton Southgate, Peterborough, England). The fluorescent stain 4',6-diamidino-2-phenylindole (DAPI), that 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 (Carl Zeiss, Jena, Germany) 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[18] and the mean value obtained. All procedures were performed on coded slides, to avoid bias.

Hybridization of tissue samples was performed following, with minor modifications, methods described by Pelissier *et al.* (2010).[19] Sections from Carnoy-fixed tissues were deparaffinized, rehydrated, post-fixed in 4% paraformaldehyde and washed. Hybridization conditions used were essentially as described above for luminal bacteria but tissue samples were incubated for 16h with the hybridization buffer. 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 15% of the pictures observed (at least 3 out of 20) was required to

decide that there was bacterial attachment to the epithelium. All procedures were performed on coded slides, to avoid bias.

mRNA Analysis

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit, (Ambion/Applied biosystems, California, USA). Thereafter, a two-step quantitative real time PCR (RT-qPCR) was performed. RNA samples were converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Only a consistent 260/280 ratio (between 1.8-2) found with NanoDrop (ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) was accepted to perform a Taqman quantitative RT-PCR. TaqMan gene expressions assays (hydrolysis probes*)* for CB1 receptors (Mm01212171_s1), CB2 receptors (Mm00438286_m1), interleukin 6 (IL-6) (Mm00446190_m1), tumor necrosis factor α (TNFα) (m00443258_m1), TPH1 (Mm00493794_m1) and TPH2 (Mm00557715_m1) were used (Applied Biosystems). β-2-microglobulin (Mm00437762_m1) was used as endogenous reference gene.

The PCR reaction mixture was incubated on the ABI 7900 HT Sequence Detection System (Applied Biosystems). All samples, as well as the negative controls, were assayed in triplicates. RQ Manager 1.2 software was used to obtain the cycle threshold for each sample; thereafter all data were analyzed with the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the vehicle – non-stressed group serving as the calibrator.[20]

Quantification of secretory immunoglobulin A

Luminal s-IgA was measured in fresh homogenates of cecal contents (diluted in PBS 1x) using a commercial double-antibody sandwich ELISA, following manufacturers' instructions (MBS564073; MyBiosource, San Diego, USA).

Histology

For histological examination, hematoxylin-eosin-stained sections from the colon were obtained following standard procedures. 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.

The mucous layer was assessed in Carnoy-fixed samples of colonic tissue. Thickness of the mucous layer was measured in 10 different fields, for triplicate, in representative regions covering, at least, 20% of the epithelial surface.[21] All measurements were performed on coded slides by two independent investigators using the Zeiss AxioVision Release 4.8.1 software. Moreover, tissue sections were also stained with Alcian Blue pH 2.5/Periodic Acid Schiff (AB 2.5/PAS kit; Bio-Optica, Milano, Italy) in order to specifically stain neutral (pink) and acidic (blue) mucins. Thereafter, colonic goblet cells were counted in 20 longitudinally-oriented villus-crypt units. Length of the villus-crypt unit was also determined to obtain goblet cells density (number of cells mm^{-1}).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was used to detect serotonin (5-HT) and Mouse Mast Cell Protease I (MMCP-I) in colonic tissue. The primary antibodies included a rabbit polyclonal anti-5-HT (1:20000; RA20080; Neuromics, Minnesota, USA) and a sheep polyclonal anti-MMCP-I (1:500; MS-RM8; Moredun, Scotland). The secondary antibodies used were a biotinylated polyclonal swine anti-rabbit IgG (1:200; E 0353; DakoCytomation, Glostrup, Denmark) or a polyclonal rabbit anti-sheep IgG-B (1:200; SC-2776, Santa Cruz Biotechnology, Santa Cruz, USA), as appropriate. Antigen retrieval for serotonin was achieved by microwave processing of the slides in 10 mM citrate buffer. Quenching of endogenous peroxidase was performed by 1-h incubation with 5% H_2O_2 in distilled water. Detection was performed with avidin/peroxidase kit (Vectastatin ABC kit; Vector Laboratories). Antigen-antibody complexes were reveled with 3-3'-diaminobenzidine (SK-4100 DAB; Vector Laboratories). Specificity of the staining was confirmed by omission of the primary antibody.

For quantification, immunopositive cells were counted at high power field (hpf; 400x magnification) in 10 microscope fields, randomly selected, in duplicate, for each tissue sample. When assessing serotonin immunoreactivity, immunopositive cells, likely corresponding to

enterochromaffin (EC) cells, were counted in the mucosa. When assessing MMCP-I immunoreactivity, immunopositive cells, corresponding to mucosal mast cells, where counted in the mucosa and submucosa. All cell counting was performed on coded slides to avoid bias.

PLASMA CORTICOSTERONE AND HAPTOGLOBIN

Plasma corticosterone levels were determined by double-antibody RIA. The characteristics of the antibody and the basic RIA procedure had been described previously.[22] In brief, 125I-corticosterone-carboximethyloxime-tyrosine-methyl ester (ICN-Biolink 2000, Barcelona, Spain), synthetic corticosterone (Sigma-Aldrich), as the standard, and an antibody raised in rabbits against corticosterone–carboximethyloxime-BSA were used. All samples were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was less than 8% and the sensitivity was 0.1 μg dL^{-1}.

Plasma concentrations of the acute-phase protein haptoglobin were determined using a commercial ELISA kit, following manufacturer's instructions (sensitivity; 0.005 mg ml^{-1}; intra-assay variability: 5.3-6.3%; inter-assay variability: 4.1-5.7%; "PHASE"^{TM} Haptoglobin Assay; Tridelta Development Limited, Maynooth, County Kildare, Ireland).

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. 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-Neuwman-Keuls multiple comparisons test. Data were considered statistically significant when $P< 0.05$. All statistical analysis were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, California, USA).

Chapter 3

RESULTS

FUNCTIONAL AND ENDOCRINE RESPONSES TO REPETITIVE PSYCHOLOGICAL STRESS (WAS)

In vehicle-treated mice maintained in non-stressful conditions pellet output was low and not affected by the antibiotic treatment (mean value for the 7 days of stress; vehicle: 3.4±0.6 faecal pellets h^{-1}; antibiotic: 3.9±0.7 faecal pellets h^{-1}; P>0.05; Fig. 1A). Repetitive WAS, independently of the experimental group considered, resulted in a significant increase in the faecal output rate during the period of stress, when compared with non-stressed groups (Fig. 1A). Defaecatory response to stress was similar in vehicle- and antibiotic-treated animals and remained stable during the seven consecutive stress sessions (Fig. 1 B).

Plasma corticosterone levels were increased in stressed animals, as assessed immediately after the last stress session. Stress-induced changes in plasma corticosterone were similar in vehicle- or antibiotic-treated mice (Fig. 1C).

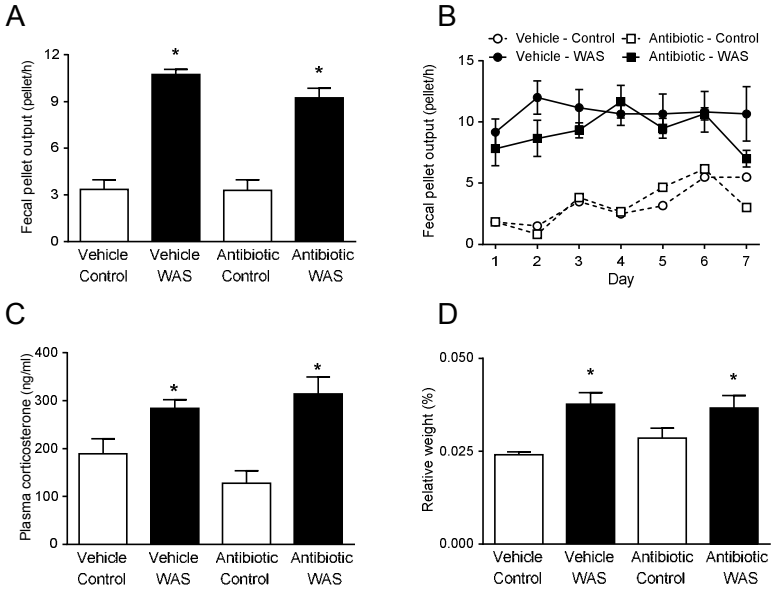


Figure 1. Functional and endocrine responses to repetitive water avoidance stress (WAS, 1 h/day for 7 days) in mice. A: Mean faecal pellet output during the time of stress, across the 7 days of treatment. *: P<0.05 vs. non-stressed groups. B: Mean faecal pellet output for the 7 WAS sessions applied. C: Plasma levels of corticosterone at the end of the last stress session. *: P<0.05 vs. non-stressed groups. D: Weight of the adrenal glands. *: P<0.05 vs. vehicle - non-stressed group; #: P<0.05 vs. antibiotic - non-stressed group. In all cases data are mean±SEM, n=6 per group.

WEIGHT OF BODY ORGANS

At necropsy, weight of the adrenal glands was increased by 50% in the vehicle-WAS group when compared with non-stressed controls (Fig. 1D). Antibiotic treatment, *per se*, resulted in a slight increase of the adrenal glands weight, without reaching statistical significance. Addition of stress in antibiotic-treated mice lead to an increase in adrenal weight similar to that observed in vehicle-treated animals (Fig. 1D). The same differences were observed for the relative weight of the adrenal glands. No consistent changes across groups were observed in the absolute or relative weight of the spleen or the thymus (Fig. 2C).

MACROSCOPIC AND MICROSCOPIC EVALUATION OF CECO-COLONIC TISSUES

In antibiotic-treated groups, irrespective of the addition of stress, the cecum appeared distended and its weight was significantly increased when compared to that of the vehicle – non-stressed group (Fig. 2A). These differences persisted when the relative weight of the cecum was calculated (data not shown), thus indicating that the increase in cecal weight was independent of a variation in body weight.

Upon macroscopical examination, both the cecum and colon showed a normal aspect, irrespective of the experimental group considered. Colonic relative weight was similar across groups (Fig. 2A). Overall, microscopic analysis of colonic tissue samples showed a normal histological structure, irrespective of the experimental group considered. Occasionally, a moderate multifocal-to-diffuse inflammatory infiltrate could be observed, but no treatment-related incidence could be established. Final histopathological scores were similar in all experimental groups (Fig. 2A). Nevertheless, total histopathological scores assigned to the antibiotic-WAS group were relatively high compared with other groups; however, no statistical significance was reached [$F(3,19)=2.090$; $P=0.135$]. Increased scores in this group were mainly associated to a worsening in the epithelial structure with increased desquamation and scant alterations in some of the crypts [$F(3,19)=3.116$; $P=0.048$ antibiotic-treated – non-stressed vs. antibiotic-treated – WAS]. No differences among groups were found in the length of the colonic crypts.

Very few MMCP-1-immunopositive cells (0 to 1 cells/field), identified as mucosal mast cells, were observed in colonic samples, irrespective of the experimental group considered (data not shown).

In Carnoy-fixed colonic samples, a layer of mucous was observed covering most of the epithelial surface. Stress decreased in a similar proportion the thickness of the mucous layer in either vehicle- or antibiotic-treated mice. Antibiotics, per se, had only a marginal, non-significant, effect reducing the thickness of the mucous layer (Table 1). Despite these changes, the density of goblet cells was similar across experimental groups. The relative abundance of mature goblet cells containing neutral mucins (pink color in a PAS/AB pH=2.5 staining) was slightly increased by either antibiotic treatment or stress, although statistical significance was only achieved for the stress group (Table 1).

Table 4. Effect of stress or/and antibiotics on the colonic mucous layer and the density of goblet cells.

	Thickness of the mucous layer (µm)	Goblet cells density(cells/mm)	Density of Mature goblet cells (cells/mm)
Vehicle – Non-stressed	23.01±1.89	99.19 ± 7.45	20.11 ± 1.90
Vehicle – WAS	11.87±0.32 *	114.06 ± 8.99	29.88 ± 0.85 *
Antibiotic – Non-stressed	17.99±0.72	113.75 ± 2.32	24.48 ± 1.32
Antibiotic – WAS	7.34±0.27 *#	111.25 ± 9.45	25.04 ± 4.6

Data are mean±SEM, n=6 animals per group. *: P<0.05 vs. vehicle – non-stressed; #: P<0.05 vs. antibiotics – non-stressed.

SYSTEMIC AND LOCAL MARKERS OF INFLAMMATION AND LUMINAL S-IGA

Plasma levels of the acute-phase protein haptoglobin were, in general, low and similar to those previously described by us.[15] No treatment-related changes in haptoblogin levels were found among groups (Fig. 2B). Similarly, no differences among groups were found for colonic cytokines mRNA expression (IL-6 and TNFα) (Fig. 2B). In most cases, there was relatively large within-group variability in the expression levels. Overall, relative expression of TNFα was higher (by 12-fold) than that of IL-6.

S-IgA was detected in all faecal samples, regardless the experimental group considered. In vehicle – non-stressed animals s-IgA levels were 7.19±1.3 µg ml^{-1}, the addition of stress increased s-IgA levels by 4.6-fold (Fig. 2D), although statistical significance was not reached. In the antibiotic

– non-stressed group s-IgA levels were increased by 36-fold (P<0.05 vs. vehicle – non-stress group). In these conditions, addition of stress did not further enhance the levels of s-IgA (Fig. 2 D).

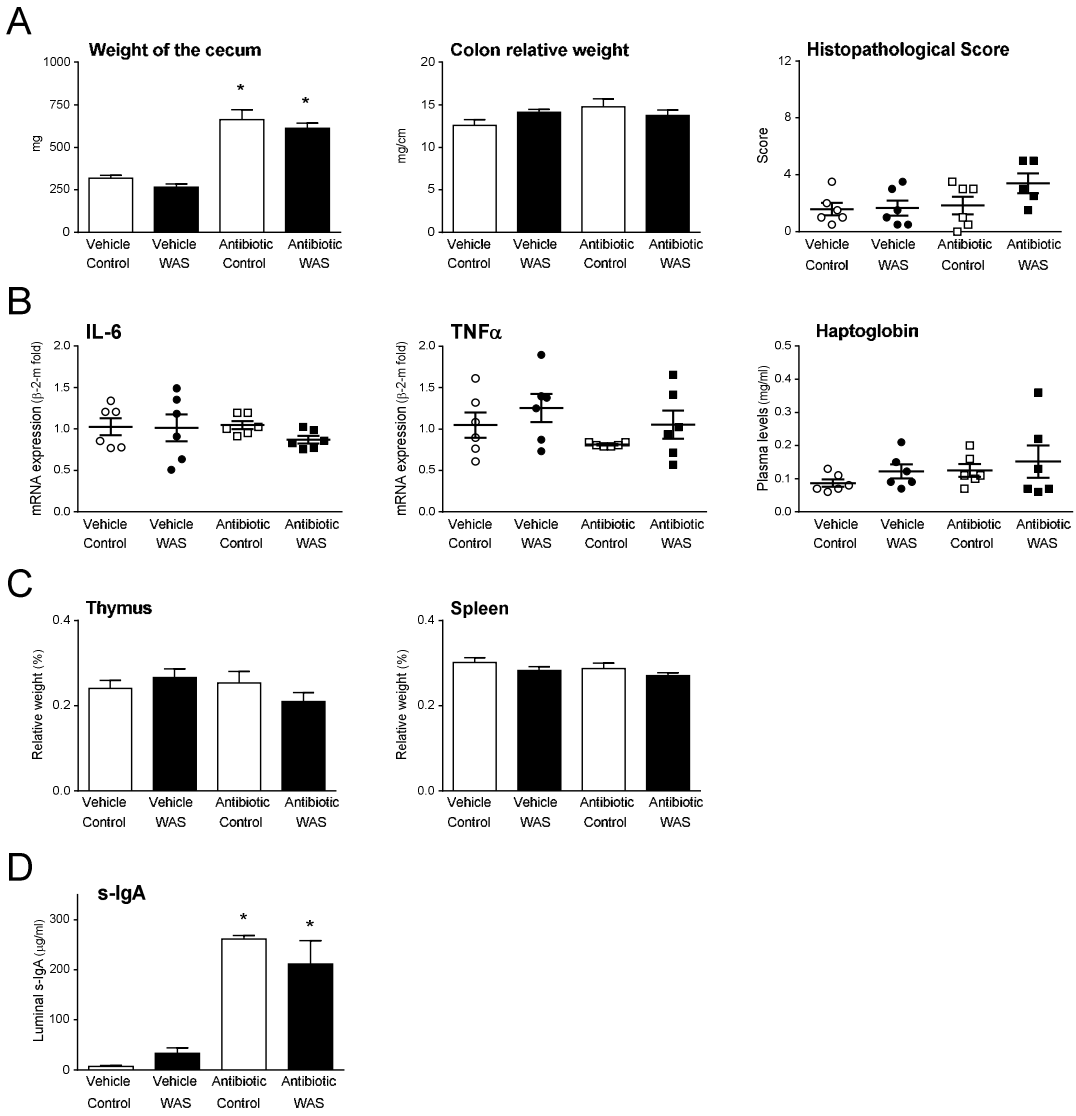


Figure 2. Ceco-colonic histopathology and immune-related parameters at the time of necropsy in the different experimental groups. A (top row): Histopathological evaluation: weight of the cecum (left panel); relative weight of the colon (middle panel) and colonic histopathological scores (right panel). Data are mean±SEM of 5-11 animals per group. Because of technical problem histopathological scores were not determined in one animal of the antibiotic-treated - WAS group. *: P<0.05 vs. vehicle – non-stressed group. B (second row): Local and systemic inflammatory markers: colonic expression of IL-6 (left panel) and TNFα (middle panel) and plasma levels of haptoglobin (right panel). Each point represents an individual animal; the horizontal bar with errors represents de mean±SEM; n=6 per group. C (third row): Relative weight (% of total body weight) of the thymus (left panel) and the spleen (right panel) Data are mean±SEM of 6 animals per group. D (bottom row): Luminal secretory IgA (s-IgA) in the different experimental groups. Data are mean±SEM of 6 animals per group. *: P< 0.05 vs. vehicle-treated groups.

CHARACTERIZATION OF LUMINAL AND WALL-ADHERED MICROBIOTA

In vehicle-treated – non-stressed animals total bacterial counts within the luminal content, determined by FISH as EUB338-positive cells, were between 3×10^{10} and 7×10^{10} cell ml^{-1}, and within the margins previously described.[21,23,24] In these conditions, EUB338 positive-bacteria represented a 90% of the total DAPI counts (Table 2). Within all bacterial groups characterized, *Bacteroides* spp. and *Clostridium* spp. were the most abundant strains; while Enterobacteria, *Lactobacillus*/*Enterococcus* spp. and *Bifidobacterium* spp. were below FISH detection levels (10^6 cell ml^{-1})[25] (Table 2; Fig. 3). Repetitive WAS had no effect, *per se,* on total bacterial counts, but induced a specific dysbiosis of the microbiota. In particular, Verrucobacteria counts were reduced to undetectable levels while counts of *Clostridium* spp. were increased by 2-fold and *Lactobacillus*/*Enterococcus* spp. appeared at a low level, borderline to the limit of detection (Table 2; Fig. 3).

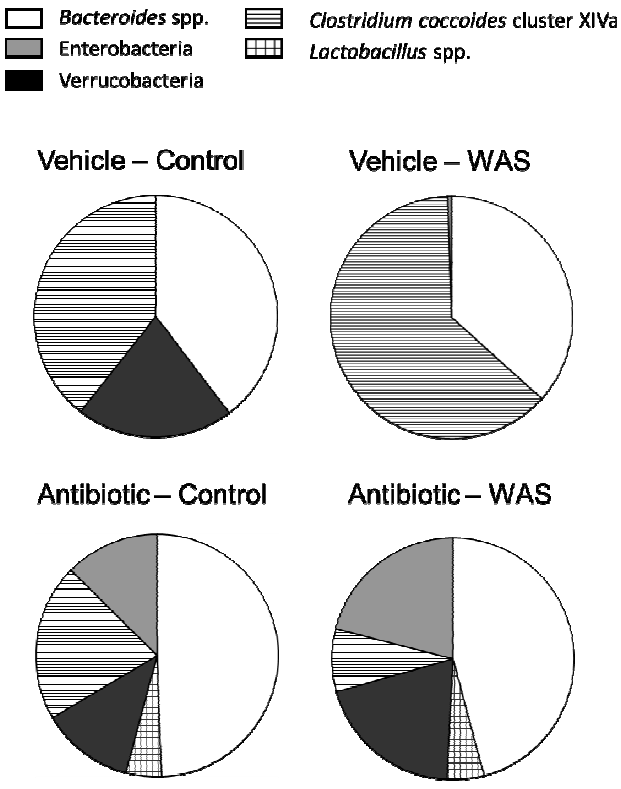


Figure 3. Relative distribution of the ceco-colonic microbiota in the different experimental groups. Data represent the relative abundance (percent) of the main bacterial groups present in the gut commensal microbiota (*Bacteroides* spp., *Clostridium* spp., Enterobacteria, *Lactobacillus* spp., and Verrucobacteria), as quantified using FISH techniques. Relative percent composition of the microbiota was calculated taking as 100% the bacterial counts obtained by FISH with the EUB 338 probe. See Table 2 for exact cell counts

Treatment with antibiotics resulted in a 2.5-fold reduction in total bacterial counts and altered the overall composition of the luminal microbiota (Table 2). In antibiotic-treated mice, EUB338 positive counts only included a 60% of the total DAPI counts. Antibiotics reduced the counts of Verrucobacteria and *Clostridium* spp., while significantly increased the counts of Enterobacteria and *Lactobacillus*/*Enterococcus* spp. (Fig. 3). Addition of stress to the antibiotic treatment further enhanced intestinal dysbiosis. In these conditions, total bacterial counts maintained their reduction when compared with the vehicle-WAS group. This was associated mainly to a 6-fold reduction in *Clostridium* spp. counts, while the counts of Verrucobacteria, Enterobacteria and *Lactobacillus*/*Enterococcus* spp. were significantly increased (Table 2; Fig. 3). *Bifidobacterium* spp. was not detected in any experimental group.

Table 5. Composition of the luminal microbiota as assessed by FISH and DAPI staining (x10^8 cells/ml).[1]

	DAPI	**Total bacteria**	***Bacteroides* spp.**	**Entero-bacteria**	**Verruco-bacteria**	***Clostridium coccoides* cluster XIVa**	***Lactobacillus – Enterococcus***	***Bifidobacterium* spp**
Vehicle – Non-stressed	462.5±56.0	433.5±27.6	69.3±3.7	ND	36.5±2.3	68.7±4.9	ND	ND
Vehicle – WAS	532.2±55.7	509.0±26.8	64.3±5.9	ND	ND	110.0±8.4$^{\&}$	0.1±0.02	ND
Antibiotic – Non-stressed	286.9±22.9	173.5±10.5*	73.7±8.4	7.0±0.6*	18.4±1.2*	31.4±1.6*$^{\#}$	18.6±1.7*	ND
Antibiotic – WAS	341.3±22.0	196.8±8.1*	103.0±9.5	11.0±0.9*$^{\#}$	44.4±1.7$^{\#}$	18.8±1.2*$^{\#}$	47.2±3.8*$^{\#}$	ND

[1]: Data represent mean±SEM from 6 animals per group. ND: Not detected (below 10^6 cells/ml). *: P<0.05 vs. vehicle – non-stressed or vehicle – WAS groups. #: P<0.05 vs. antibiotic – non-stressed group. &: P<0.05 vs. vehicle – non-stressed group

As it relates to bacterial wall adherence, EUB-338-positive cells were always observed attached to the wall, in most cases within the mucous layer located on the epithelial surface. In vehicle-non-stressed animals, the only bacterial group attached to the colonic wall was

Verrucobacteria (Incidence: 83%). Addition of stress significantly reduced the incidence of Verrucobacteria attachment (Table 3, Fig. 4), without affecting the adherence of other bacterial groups.

Table 6. Incidence of bacterial wall adherence.[1]

	Bacteroides spp.	**Enterobacteria**	**Verrucobacteria**	**Clostridium coccoides cluster XIVa**	**Lactobacillus-Enterococcus spp**
Vehicle – Non-stressed	0/6 (0%)	0/6 (0%)	5/6 (83 %)	1/6 (17 %)	0/6 (0%)
Vehicle – WAS	0/6 (0%)	0/6 (0%)	1/6 (17 %)	2/6 (33 %)	0/6 (0%)
Antibiotic – Non-stressed	0/6 (0%)	6/6 (100 %)	5/6 (83 %)	5/6 (83 %)	5/6 (83 %)
Antibiotic – WAS	3/6 (50 %)	2/6 (33 %)	5/6 (83 %)	6/6 (100 %)	5/6 (83 %)

[1]: Data represent the number of animals showing bacterial wall adherence over the total of animals (percentage of incidence).

During antibiotic treatment, the incidence of bacterial wall adherence increased significantly for all bacterial groups detected in the luminal content (83-100% incidence), except for *Bacteroides* spp. (0% incidence). The addition of stress maintained a generalized adherence for all groups explored, but, particularly, facilitated *Bacteroides* spp. attachment while reduced the adherence of Enterobacteria (Table 3, Fig. 4).

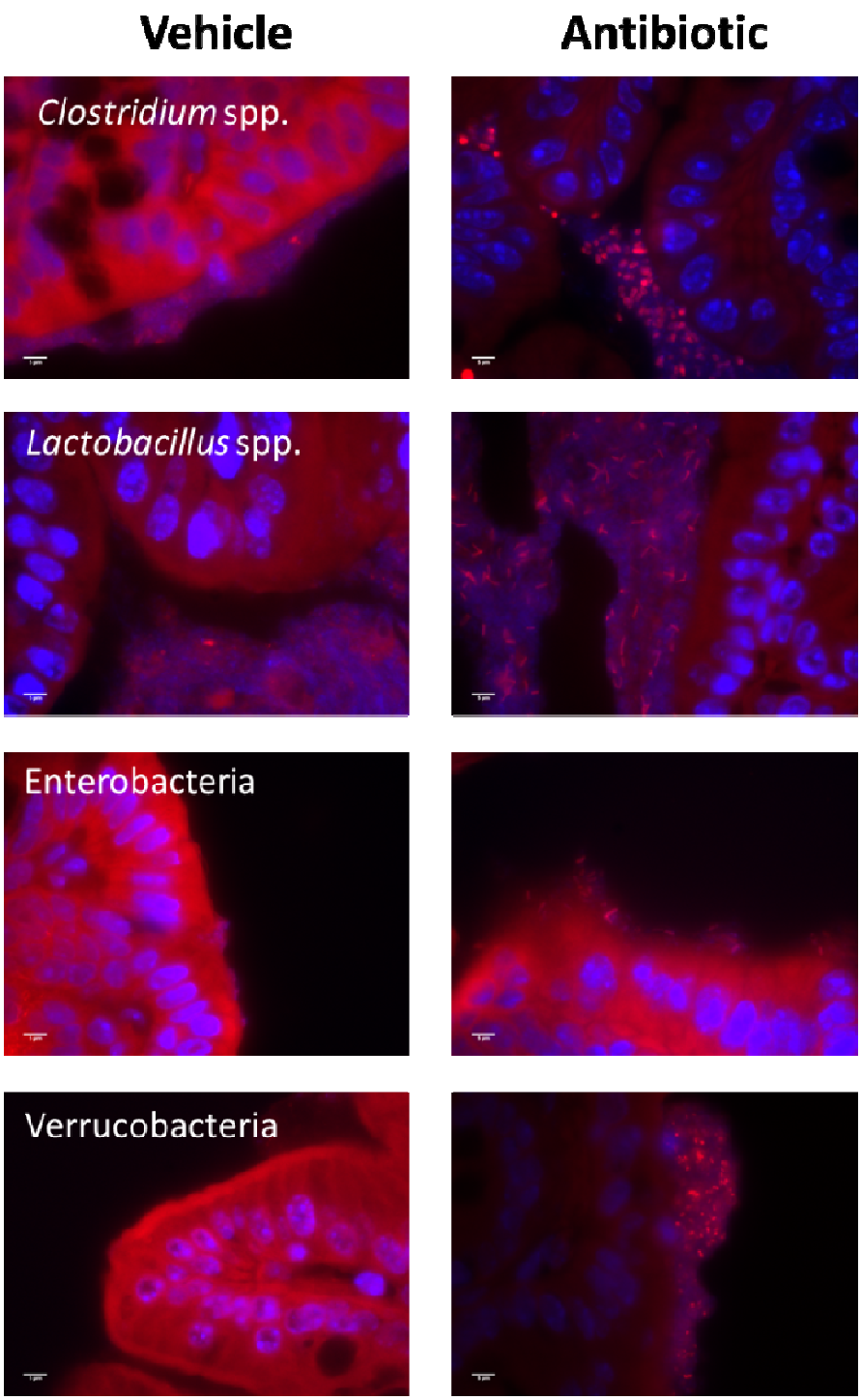


Figure 4. Representative colonic tissue images showing bacterial wall adherence for different bacterial groups. The left column corresponds to a vehicle-treated mice and the right column to an antibiotic-treated animal. Each line corresponds to a different bacterial group (from top to bottom: *Clostridium* spp, *Lactobacillus* spp, Enterobacteria and Verrucobacteria). Note the higher abundance of bacteria attached to the epithelium and within the mucous layer covering the epithelial surface in the antibiotic-treated mice compared with the vehicle-treated mice.

EXPRESSION OF CANNABINOID RECEPTORS AND ACTIVITY OF THE SEROTONERGIC SYSTEM

mRNA for both cannabinoid receptors was detected in all samples. Expression levels in control conditions (vehicle – non-stressed animals) were low, with the levels of CB1 mRNA being about 10-fold higher than those of CB2.In vehicle-treated mice, WAS had a marginal effect increasing CB2 expression (by 6%, $P>0.05$). In the antibiotic – non-stress group, CB2 expression was increased by 20% ($P<0.05$ vs. vehicle – non-stress group); addition of stress further increased CB2 expression, leading, approximately, to a 40% increase in expression ($P<0.05$ vs. vehicle-treated groups; Fig 5A). CB1 expression was not affected by either stress or antibiotics, alone or in combination (Fig 5A). Regardless of the experimental group considered, expression levels of CB2 receptors correlated positively with *Lactobacillus* spp. counts ($P=0.001$; $r^2=0.38$) and negatively with *Clostridium* spp. counts ($P=0.02$; $r^2=0.21$) (Fig. 5B).

The isoform 1 of the TPH was detected with high reproducibility and at relatively high levels in colonic tissues (Fig. 5C); however the isoform 2 (TPH2) was found in a very low quantity (a mean of 36.6 Cq value). Overall, TPH1 expression levels were about 24-fold higher than those of TPH2. TPH1 expression levels were similar in vehicle-treated- or antibiotic-treated non-stressed animals. Repetitive WAS increased TPH1 expression by similar proportion in either vehicle- or antibiotic-treated animals (40% increase; Fig. 5C).

5-HT-immunopositve cells, likely corresponding to EC cells, were scattered throughout the colonic mucosa. Relative abundance was similar in all experimental groups (Fig. 5C).

BEHAVIORAL RESPONSES TO INTRACOLONIC CAPSAICIN

Intracolonic administration of capsaicin induced pain-related behaviors in all mice during the 30 min observation period. The behavior more expressed was the liking of the abdominal area, which was observed in all animals. In the vehicle – non-stress group the number of pain-related behaviours reached a mean value of 40.9±6.6 in the 30 min observation period (n=5; Fig. 5D). In these conditions, treatment with antibiotics slightly reduced the number of pain behaviours, although statistical significance was not reached. In vehicle-treated animals, addition of stress increased the incidence of behaviors by 48% ($P=0.071$ vs. vehicle – non-stress group) an effect completely prevented by the treatment with antibiotics ($P<0.05$ vs. vehicle – stress group; Fig. 5D)

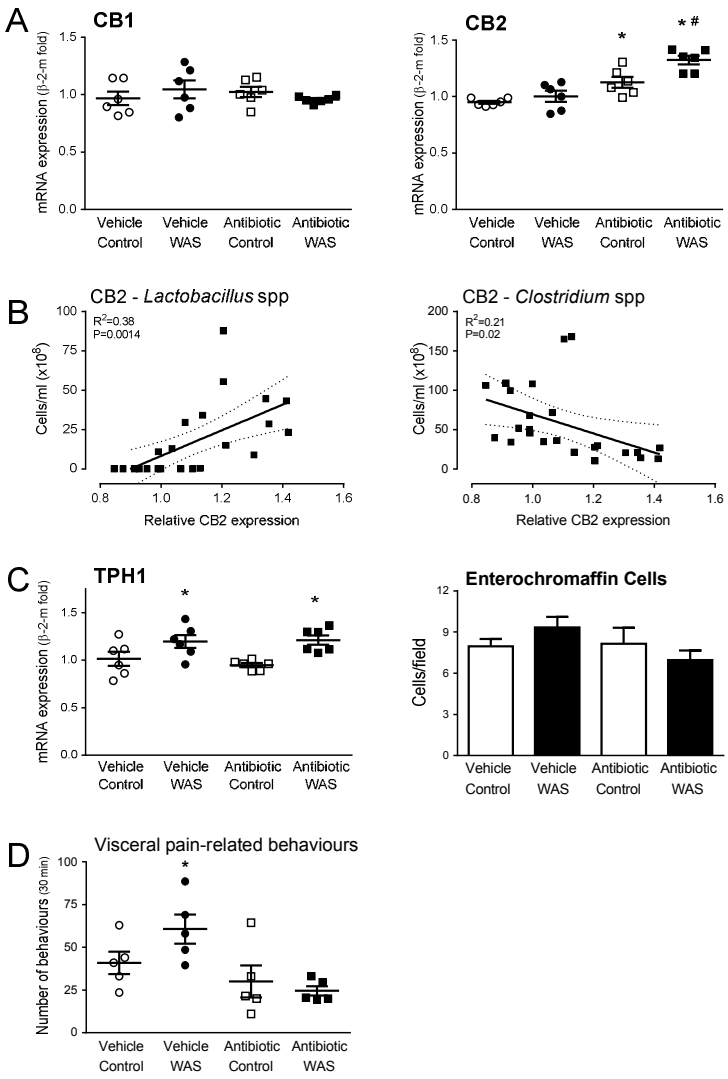


Figure 5. Effects of stress and/or antibiotics on the colonic sensory-related systems. A (upper row): Colonic expression of cannabinoid receptors, CB1 (left pane) and CB2 (right panel) in the different experimental groups. Each point represents an individual animal; the horizontal bar with errors represents de mean±SEM of each group; n=5-6 per group. *: P<0.05 vs. vehicle – non-stressed and vehicle – WAS groups. #: P<0.05 vs. antibiotic – non-stressed group. B (second row): Correlations between the relative expression of CB2 and the bacterial counts of Lactobacillus spp. (P=0.0014; r2=0.38) (left panel) and Clostridium spp. (P=0.02; r2=0.21) (right panel), as determined by FISH. Each point represents an individual animal; broken lines represent the 95% confidence interval. C (thirst row): Activity of the serotonergic system within the colon. Left panel shows the relative expression of tryptophan hydroxylase 1 (TPH1). Each point represents an individual animal; the horizontal bar with errors represents the mean±SEM of each group; n=6 per group. *: P<0.05 vs. respective non-stressed group. Right panel show the density of enterochromaffin cells (5-HT-immunorreactive cells/field, X400), as determined by immunohistochemistry, in the different experimental groups. Data are mean±SEM of n=6 animals per group. D (lower row): Total number of capsaicin-induced behaviors (right panel) during 30 minutes in the different experimental groups. Each point represents an individual animal; the horizontal bar with errors represents de mean±SEM of each group; n=5 per group. *: P<0.05 vs. vehicle – WAS groups.

DISCUSSION

In this study we show that the colonic functional (motor) and endocrine responses to stress are essentially not affected by relatively large alterations of the ceco-colonic microbiota, either luminal or attached to the colonic wall, during an antibiotic treatment. Moreover, we show that microbiological changes, due to antibiotics and stress, are able to modulate the immune and sensory systems, namely the endocannabinoid and the serotonergic systems, within the colon, without the induction of a manifest state of intestinal inflammation. In dysbiotic animals visceral pain-related responses were reduced, thus suggesting that these modulatory effects on sensory systems might have functional consequences, leading to a hypoalgesic-like sate.

Our results confirm the validity of chronic WAS as a valid, mild stressor in mice, as previously published.[14,15,26] Mice did not habituate to the stress protocol, as shown by the persistent colonic response along the 7-day period of WAS. Moreover, the efficacy of the stress paradigm is further demonstrated by the raise in plasma corticosterone and the increase in weight of the adrenal glands at the end of the last stress session.

Total bacterial counts were not affected by stress. However, repetitive WAS significantly increased the counts of *Clostridium* spp. and favored the appearance of *Lactobacillus* spp. These changes agree with those described in mice subjected to social stress, where the main change in the microbiota was an increase in the Clostridia group.[13] Interestingly, the Verrucobacteria group, present in a relatively high proportion in non-stressed mice, was undetectable in stressed animals. This group of microorganisms, which degrade mucus within the gastrointestinal tract,[17,27] might have relevance in gastrointestinal diseases. For instance, an enhancement of the mucin-degrading microbiota in dysbiotic patients predispose to Crohn's Disease.[28] During stress, the thickness of the mucus layer was reduced, in agreement with O'Malley *et al.* (2010).[28,29] A reduction in mucus abundance might be a factor reducing also the relative abundance of Verrucobacteria. Alternatively, we cannot discard that these changes are secondary to the combined enhancing effects of stress on colonic motility and mucus secretion,[30-32] leading to an increased discharge of mucus and therefore to a net reduction in mucus content and associated bacteria. Moreover, although goblet cell density remained stable, stress increased the proportion of mature goblet cells, indicative of an increase in mucus production and secretion.[33] Despite these changes in mucus content, wall adhered microbiota was not affected by stress.

As expected, treatment with wide-spectrum, non-absorbable antibiotics significantly reduced total bacterial counts. The reduction in bacterial counts was coupled to a specific dysbiosis which implied a proliferation of *Lactobacillus* spp. and Enterobacteria; while the *Clostridium* spp. and the Verrucobacteria groups were reduced. Interestingly, only antibiotic-induced changes in luminal microbiota were associated to an increase in bacterial wall adherence. This is important because adhered microbiota has been suggested to be the one directly interacting with the host's bacterial recognition systems, thus eliciting either beneficial or harmful responses within the gut.[34,35] The relationship between luminal counts and epithelial attachment seems to be strain-dependent. Overall, changes in bacterial wall adherence correlated positively with changes in luminal counts. However, the Clostridia group was reduced during antibiotic treatment, but presented an increased rate of adherence. This negative relationship might reflect the heterogeneity of *Clostridium coccoides* cluster XIVa. From the present data, we cannot rule out the possibility that antibiotics are affecting only a part of this cluster, leading to a relative selection of bacteria with high wall-adherence capacities. In fact, it is well reported that most antibiotics can increase the risk of developing *Clostridium difficile* colitis[36,37] and that the relapse of colitis in patients with recurrent *C. difficile* infections is associated with reduced intestinal microbial diversity.[38] Nevertheless, the role of gut commensal microbiota in intestinal inflammation remains controversial, and beneficial effects of wide spectrum antibiotics has been shown in DSS-induced colitis in rats.[39]The mucous layer represents also a protective barrier preventing bacterial wall adherence. Therefore, a loss of mucus should be regarded as a factor favoring bacterial-host interactions.[40,41] Antibiotics had only a marginal effect reducing the mucous layer, thus suggesting that the mucus, *per se*, might play a minor role affecting bacterial wall adherence in the present conditions. Ceco-colonic dysbiosis was further enhanced when antibiotic-treated mice were subjected to stress. This was associated to a significant increase in the incidence of wall adherence, observed for all bacterial groups assessed, and a clear reduction in the thickness of the mucus layer.

Commensal microbiota is necessary for the development of spontaneous colitis, as suggested by observation in mice deficient in interleukin 10; however, gut commensal microbiota could also have a protective role, as seen in germ-free mice with DSS-induced colitis.[42-44] These apparent discrepancies might be associated to the composition of the microbiota, the immaturity of the immune system, the environmental conditions of housing and the type of treatment applied (duration and antibiotics used). In any case, the potential pathophysiological implications of these

observations warrant further investigations. In humans, increased bacterial wall adherence has been suggested as a pathogenic factor leading to local immune responses that favor the appearance and maintenance of intestinal inflammation.[21,45] Interestingly, antibiotic-induced dysbiosis had no impact on the gut-to-brain modulation of endocrine responses to psychological stress. This agrees with recent data suggesting that the gut-to-brain signaling is established during the early post-natal phase and that commensal microbiota is important during that imprinting period.[4,46] Once the gut is colonized and the commensal microbiota established, changes in microbiota composition seem to have a minor impact in gut-to-brain signaling, at least as stress-related endocrine responses relates.[46] Despite this, intestinal microbiota has been related as a putative factor affecting gut sensory systems leading to altered behavioral[47,48] and local visceral responses, such as visceral pain.[12,49]For instance, gut commensal microbiota is fundamental for the development of inflammatory pain in mice.[12,49,50] Here, we assessed changes in the endocannabinoid and the serotonergic systems, two of the main sensory systems within the gut, with a demonstrated involvement in secretomotor- and visceral pain-related responses.[49,51-54] In the present conditions, antibiotics selectively up-regulated the expression of CB2; an effect further enhanced by the addition of stress. This agrees with data suggesting that gut microbiota is able to up-regulate the endocannabinoid system within the gut.[55] Modification of the commensal microbiota by addition of specific bacterial strains (namely *L. acidophilus*) has been shown to up-regulate CB2 expression in rats and mice, leading to the induction of visceral analgesia.[49] In agreement with this, changes in CB2 expression correlated positively with luminal counts of *Lactobacillus* spp., which increased with antibiotic treatment and were further enhanced in stressed antibiotic-treated mice. Overall, these observations further support the view that bacteria of the *Lactobacillus* spp. group should be regarded as a beneficial component of the microbiota, which might be implicated in the modulation of visceral pain responses through the modulation of the intestinal endocannabinoid system. On the other hand, counts of *Clostridium* spp. correlated in a negative manner with the CB2 expression reinforcing the potential role assigned to this bacterial group as a pathogenic component of the microbiota.

Expression of TPH1 and TPH2 and density of EC cells served to assess the activity of the serotonergic system. As expected, expression of TPH2, the isoform responsible for the synthesis of neuronal serotonin, was very low in whole colonic homogenates. On the other hand, expression TPH1, responsible for serotonin synthesis in EC cells, was detected at relatively high levels. Interestingly, TPH1 was up-regulated in stressed animals, independently of the antibiotic

treatment. These observations might suggest that, although not directly assessed, serotonin synthesis and availability is increased during stress, with commensal microbiota playing a minor role *per se*. Overall, this agrees with studies showing that serotonin availability might be increased within the colon during stress.[56] However, density of EC cells was not affected by stress, thus suggesting a cellular hyperactivity, rather than a hyperplasia. This contrasts with inflammatory models of gut dysfunction, such as the experimental infection with *Trichinella spiralis*, in which increased availability of serotonin has been associated to a hyperplasia of EC cells.[57,58] The functional consequences of these changes in the cannabinoid and serotonergic systems warrant further studies, outside the original scope of the present work.

The changes observed in the expression of sensory-related systems are likely to have a functional significance. This is demonstrated by the changes in visceral pain-related responses observed in antibiotic-treated vs. non-treated animals. In agreement with previous reports, we show that intracolonic capsaicin evokes behavioral responses consistent with the induction of visceral pain.[9,10] An increase in pain-related events was observed in stressed animals, thus confirming data indicating that repeated psychological stress induces visceral hypersensitivity in rodents.[59] Interestingly, stress-induced hyperalgesic responses were completely prevented by the treatment with antibiotics. These observations might suggest that the changes in CB2 expression and serotonin availability might lead to functional effects modulating visceral sensitivity. Similarly, other sensory mediators not directly assessed here and involved in visceral pain responses, such as vanilloids,[60] might be involved in the responses observed. Overall, these observations further support an involvement of gut microbiota as a modulatory component of gut sensory functions.

As mentioned, none of the treatments applied resulted in evident intestinal inflammation. Although enlargement of the cecum was observed in antibiotic-treated animals, this was not associated to consistent histopathological alterations. It is interesting to point out that despite the increased host-bacterial interaction observed in dysbiotic mice, no signs of colonic inflammation (either macroscopical, microscopical or biochemical) were observed following the treatment with antibiotics. This contrasts with previous reports that observed signs of intestinal inflammation during both antibiotic treatment and stress.[12,41,61,62] In particular, the appearance of stress-induced intestinal inflammation has been related with a mast cell infiltrate and the facilitation of bacterial wall adherence in rats.[12,41,61,62] However, in our conditions, the density of mast cells was not increased by stress. Although inflammatory markers were unaltered luminal s-IgA levels were

increased during dysbiosis. Luminal s-IgA contributes to the suppression of immune reactions generated by commensal bacteria [63,64] and, when binding to bacteria, prevent bacterial translocation.[65] Increased s-IgA levels might represent a mucosal response, likely triggered by the increased rate of bacterial attachment during dysbiosis, aiming the prevention of local and systemic inflammation and bacterial translocation. Multiple factors ranging from the species/strain used to the intensity of the stressors applied or the microbial environment might contribute to the final immune response to a dysbiotic state. Systematic studies addressing these aspects will be necessary to determine the relative contribution of these factors to the final responses observed within the gut.

In summary, the current study shows that gut commensal microbiota and stress are likely to act as interactive components in the maintenance of gut homeostasis and in the development of gut pathophysiology. Changes observed here suggest that microbiota and stress are able to selectively modulate gut sensory mechanisms, in the absence of obvious structural or biochemical alterations compatible with the presence of intestinal inflammation. Nevertheless, a mucosal immune response, characterized by increased s-IgA production, could be observed. Moreover, dysbiosis was associated to a reduction in visceral pain-related responses, thus suggesting that microbiota, influencing sensory-related systems within the gut, is able to modulate visceral pain arising from the gut. Overall, these data support the potential involvement of stress and gut microbiota in the alterations observed in patients with functional gastrointestinal disorders, characterized by secretomotor and sensory alterations in the absence of structural changes. These observations warrant further studies dissecting the pathways altered by stress and gut microbes and the associated functional changes. Moreover, our observations support the view that the beneficial effect of certain bacterial strains, used as probiotics, might be associated to the modulation of the activity of endogenous sensory-related systems, such as the endocannabinoid system.

ACKNOWLEDGEMENTS

E. Martínez and A. Acosta are thanked for their technical assistance. This study was supported by grants BFU2009-08229 and BES-2010-037699 (FPI program; M. A. personal support) from the Ministerio de Ciencia e Innovación (Spain) and 2009SGR708 from the Generalitat de Catalunya.

The authors don't have any conflict of interest.

REFERENCES

1. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology 2006; 130:1480-1491.

2. Elsenbruch S. Abdominal pain in Irritable Bowel Syndrome: a review of putative psychological, neural and neuro-immune mechanisms. Brain Behav Immun 2011; 25:386-394.

3. Barbara G, Cremon C, De Giorgio R, Dothel G, Zecchi L, Bellacosa L, et al. Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. Curr Gastroenterol Rep 2011; 13:308-315.

4. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM, et al. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 2009; 65:263-267.

5. Bercik P, Collins SM, Verdu EF. Microbes and the gut-brain axis. Neurogastroenterol Motil 2012; 24:405-413. .

6. DuPont AW, DuPont HL. The intestinal microbiota and chronic disorders of the gut. Nat Rev Gastroenterol Hepatol 2011; 8:523-531.

7. Mantis NJ, Rol N, Corthesy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 2011; 4:603-611.

8. Hansen J, Gulati A, Sartor RB. The role of mucosal immunity and host genetics in defining intestinal commensal bacteria. Curr Opin Gastroenterol 2010; 26:564-571.

9. van der Waaij D, Berghuis-de Vries JM, Korthals Altes C. Oral dose and faecal concentration of antibiotics during antibiotic decontamination in mice and in a patient. J Hyg (Lond) 1974; 73:197-203.

10. Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, et al. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 2006; 55:182-190.

11. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: Implications for stressor-induced immunomodulation. Brain Behav Immun 2011; 25:397-407.

12. Larsson MH, Miketa A, Martinez V. Lack of interaction between psychological stress and DSS-induced colitis affecting colonic sensitivity during colorectal distension in mice. Stress 2009; 12:434-444.

13. Melgar S, Engstrom K, Jagervall A, Martinez V. Psychological stress reactivates dextran sulfate sodium-induced chronic colitis in mice. Stress 2008; 11:348-362.

14. Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F. A new model of visceral pain and referred hyperalgesia in the mouse. Pain 2001; 92:335-342.

15. Teran-Ventura E, Roca M, Martin MT, Abarca ML, Martinez V, Vergara P. Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats. Microb Ecol 2010; 60:691-702.

16. Salzman NH, de Jong H, Paterson Y, Harmsen HJ, Welling GW, Bos NA. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiology 2002; 148:3651-3660.

17. Selinummi J, Seppala J, Yli-Harja O, Puhakka JA. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. BioTechniques 2005; 39:859-863.

18. Pelissier MA, Vasquez N, Balamurugan R, Pereira E, Dossou-Yovo F, Suau A, et al. Metronidazole effects on microbiota and mucus layer thickness in the rat gut. FEMS Microbiol Ecol 2010; 73:601-610.

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-408.

20. Swidsinski A, Loening-Baucke V, Theissig F, Engelhardt H, Bengmark S, Koch S, et al. Comparative study of the intestinal mucus barrier in normal and inflamed colon. Gut 2007; 56:343-350.

21. Amador-Arjona A, Delgado-Morales R, Belda X, Gagliano H, Gallego X, Keck ME, et al. Susceptibility to stress in transgenic mice overexpressing TrkC, a model of panic disorder. J Psychiatr Res 2010; 44:157-167.

22. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2007; 2:204.

23. Swidsinski A, Sydora BC, Doerffel Y, Loening-Baucke V, Vaneechoutte M, Lupicki M, et al. Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. Inflamm Bowel Dis 2007; 13:963-970.

24. Zoetendal EG, Mackie RI. Molecular methods in microbial ecology. In: Tannock GW, editor. Probiotics and prebiotics: Scientific aspects. 1st ed. Dunedin, New Zealand: Caister Academic Press, University of Otago; 2005. pp. 1-24.

25. Larauche M, Gourcerol G, Million M, Adelson DW, Tache Y. Repeated psychological stress-induced alterations of visceral sensitivity and colonic motor functions in mice: influence of surgery and postoperative single housing on visceromotor responses. Stress 2010; 13:343-354.

26. Derrien M, Collado MC, Ben-Amor K, Salminen S, de Vos WM. The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract. Appl Environ Microbiol 2008; 74:1646-1648.

27. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut 2011; 60:631-637.

28. O'Malley D, Julio-Pieper M, Gibney SM, Dinan TG, Cryan JF. Distinct alterations in colonic morphology and physiology in two rat models of enhanced stress-induced anxiety and depression-like behaviour. Stress 2010; 13:114-122.

29. Martinez V, Wang L, Rivier J, Grigoriadis D, Tache Y. Central CRF, urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. J Physiol 2004; 556:221-234.

30. Soderholm JD, Perdue MH. Stress and gastrointestinal tract. II. Stress and intestinal barrier function. Am J Physiol Gastrointest Liver Physiol 2001; 280:G7-G13.

31. Caso JR, Leza JC, Menchen L. The effects of physical and psychological stress on the gastro-intestinal tract: lessons from animal models. Curr Mol Med 2008; 8:299-312.

32. Bergstrom KS, Guttman JA, Rumi M, Ma C, Bouzari S, Khan MA, et al. Modulation of intestinal goblet cell function during infection by an attaching and effacing bacterial pathogen. Infect Immun 2008; 76:796-811.

33. Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S. The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol Rev 2011; 35:681-704.

34. Salzman NH. Microbiota-immune system interaction: an uneasy alliance. Curr Opin Microbiol 2011; 14:99-105.

35. Bishara J, Peled N, Pitlik S, Samra Z. Mortality of patients with antibiotic-associated diarrhoea: the impact of Clostridium difficile. J Hosp Infect 2008; 68:308-314.

36. Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, et al. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect Immun 2012; 80:62-73.

37. Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, et al. Decreased diversity of the fecal Microbiome in recurrent Clostridium difficile-associated diarrhea. J Infect Dis 2008; 197:435-438.

38. Videla S, Vilaseca J, Guarner F, Salas A, Treserra F, Crespo E, et al. Role of intestinal microflora in chronic inflammation and ulceration of the rat colon. Gut 1994; 35:1090-1097.

39. Schultsz C, Van Den Berg FM, Ten Kate FW, Tytgat GN, Dankert J. The intestinal mucus layer from patients with inflammatory bowel disease harbors high numbers of bacteria compared with controls. Gastroenterology 1999; 117:1089-1097.

40. Soderholm JD, Yang PC, Ceponis P, Vohra A, Riddell R, Sherman PM, et al. Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. Gastroenterology 2002; 123:1099-1108.

41. Guarner F, Malagelada JR. Role of bacteria in experimental colitis. Best Pract Res Clin Gastroenterol 2003; 17:793-804.

42. Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. Gut Microbes 2011; 2:145-158.

43. Tlaskalova-Hogenova H, Stepankova R, Kozakova H, Hudcovic T, Vannucci L, Tuckova L, et al. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol 2011; 8:110-120.

44. Martinez-Medina M, Aldeguer X, Gonzalez-Huix F, Acero D, Garcia-Gil LJ. Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. Inflamm Bowel Dis 2006; 12:1136-1145.

45. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 2004; 558:263-275.

46. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 2011; 108:16050-16055.

47. Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ, et al. Bacterial infection causes stress-induced memory dysfunction in mice. Gut 2011; 60:307-317.

48. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 2007; 13:35-37.

49. Amaral FA, Sachs D, Costa VV, Fagundes CT, Cisalpino D, Cunha TM, et al. Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 2008; 105:2193-2197.

50. Brusberg M, Arvidsson S, Kang D, Larsson H, Lindstrom E, Martinez V. CB1 receptors mediate the analgesic effects of cannabinoids on colorectal distension-induced visceral pain in rodents. J Neurosci 2009; 29:1554-1564.

51. Cremon C, Carini G, Wang B, Vasina V, Cogliandro RF, De Giorgio R, et al. Intestinal serotonin release, sensory neuron activation, and abdominal pain in irritable bowel syndrome. Am J Gastroenterol 2011; 106:1290-1298.

52. Yuce B, Kemmer M, Qian G, Muller M, Sibaev A, Li Y, et al. Cannabinoid 1 receptors modulate intestinal sensory and motor function in rat. Neurogastroenterol Motil 2010; 22:672-e205.

53. Zoppi S, Madrigal JL, Perez-Nievas BG, Marin-Jimenez I, Caso JR, Alou L, et al. Endogenous Cannabinoid System Regulates Intestinal Barrier Function in Vivo through Cannabinoid Type 1 Receptor Activation. Am J Physiol Gastrointest Liver Physiol 2011; 302:G565-571.

54. Muccioli GG, Naslain D, Backhed F, Reigstad CS, Lambert DM, Delzenne NM, et al. The endocannabinoid system links gut microbiota to adipogenesis. Mol Syst Biol 2010; 6:392.

55. Julio-Pieper M, O'Mahony CM, Clarke G, Bravo JA, Dinan TG, Cryan JF. Chronic stress-induced alterations in mouse colonic 5-HT and defecation responses are strain dependent. Stress 2012; 15:218-226.

56. Bertrand PP, Barajas-Espinosa A, Neshat S, Bertrand RL, Lomax AE. Analysis of real-time serotonin (5-HT) availability during experimental colitis in mouse. Am J Physiol Gastrointest Liver Physiol 2010; 298:G446-455.

57. Wheatcroft J, Wakelin D, Smith A, Mahoney CR, Mawe G, Spiller R. Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction. Neurogastroenterol Motil 2005; 17:863-870.

58. Reber SO. Stress and animal models of inflammatory bowel disease--an update on the role of the hypothalamo-pituitary-adrenal axis. Psychoneuroendocrinology 2012; 37:1-19.

59. Santos J, Yang PC, Soderholm JD, Benjamin M, Perdue MH. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. Gut 2001; 48:630-636.

60. Tsuruta T, Inoue R, Iwanaga T, Hara H, Yajima T. Development of a method for the identification of S-IgA-coated bacterial composition in mouse and human feces. Biosci Biotechnol Biochem 2010; 74:968-973.

61. Mathias A, Corthesy B. N-Glycans on secretory component: mediators of the interaction between secretory IgA and gram-positive commensals sustaining intestinal homeostasis. Gut Microbes 2011; 2:287-293.

62. Duerkop BA, Vaishnava S, Hooper LV. Immune responses to the microbiota at the intestinal mucosal surface. Immunity 2009; 31:368-376.

.

Chapter 4

CHAPTER 4

ANTIBIOTIC-INDUCED DYSBIOSIS ALTERS HOST-BACTERIAL INTERACTIONS AND LEADS TO COLONIC SENSORY AND MOTOR CHANGES IN MICE

M. Aguilera[a,b], M. Cerdà-Cuéllar[c,d] and V. Martínez[a,b,e]

[a]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain.

[b]Neuroscience Institute, Universitat Autònoma de Barcelona, Barcelona, Spain.

[c]Centre de Recerca en Sanitat Animal (CReSA), Universitat Autònoma de Barcelona, Barcelona, Spain.

[d]Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain.

[e]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Spain.

Chapter 4

ABSTRACT

Alterations in the composition of the commensal microbiota (dysbiosis) seem to be a pathogenic component of functional gastrointestinal disorders, mainly irritable bowel syndrome (IBS), and might participate in the secretomotor and sensory alterations observed in these patients.

We determined if a state antibiotics-induced intestinal dysbiosis is able to modify colonic pain-related and motor responses and characterized the neuro-immune mechanisms implicated in mice.

A 2-week antibiotics treatment induced a colonic dysbiosis (increments in *Bacteroides* spp, *C. coccoides* and *Lactobacillus* spp and reduction in *Bifidobacterium* spp). Bacterial adherence was not affected. Dysbiosis was associated to increased levels of secretory-IgA, up-regulation of the antimicrobial lectin RegIIIγ, and toll-like receptors (TLR) 4 and 7 and down-regulation of the antimicrobial-peptide Resistin-Like Molecule-β and TLR5. Dysbiotic mice showed less goblet cells, without changes in the thickness of the mucus layer. Neither histological nor molecular signs of inflammation were observed. In dysbiotic mice, expression of the cannabinoid receptor 2 was up-regulated, while the cannabinoid 1 and the mu-opioid receptors were down-regulated. In antibiotic-treated mice, visceral pain-related responses elicited by intraperitoneal acetic acid or intracolonic capsaicin were significantly attenuated. Colonic contractility was enhanced during dysbiosis.

Intestinal dysbiosis induce changes in the innate intestinal immune system and modulate the expression of pain-related sensory systems, an effect associated with a reduction in visceral pain-related responses. Commensal microbiota modulates gut neuro-immune sensory systems, leading to functional changes, at least as it relates to viscerosensitivity. Similar mechanisms might explain the beneficial effects of antibiotics or certain probiotics in the treatment of IBS.

Keywords: Cannabinoid receptors; Colonic motility; Gut commensal microbiota; Innate immune system; Intestinal dysbiosis, Opioid receptors; Visceral sensitivity

INTRODUCTION

Functional gastrointestinal disorders (FGDs) are highly prevalent alterations characterized by an altered gastrointestinal (GI) functionality in the absence of overt structural changes. Although FGDs might affect any segment of the GI tract, most of the patients present symptoms related to lower GI (colon) dysfunction, and are grouped as irritable bowel syndrome (IBS) patients. Main IBS symptoms include abdominal pain or discomfort, bloating, abdominal distension and altered bowel habits.[1] Although still partially unknown, IBS has a multifactorial pathogenesis involving psychosocial factors (such as stress), an intestinal immune activation (with a persistent low grade inflammation) and altered brain-gut-brain communication and host-microbial interactions.[1,2]

Within the intestine, microbial community is established shortly from birth and acts as an entire organ.[3,4] Recent works have identified gut commensal microbiota (GCM) as a dynamic ecosystem that maintains a bidirectional relationship with the host and that is essential for physiological and pathophysiological states.[5–8] Within the GI tract, GCM has a distinct distribution, with the higher bacterial counts localized in the more distal areas (large intestine). Colonic microbiota is composed mainly by microorganisms from the Firmicutes and Bacteroidetes phyla (mainly *Clostridium* spp, *Lactobacillus* spp and Segmented Filamentous Bacteria), sharing the colonic niche with less abundant bacteria from the Actinobacteria and Proteobacteria phyla (mainly *Bifidobacterium* spp, Verrucobacteria and Enterobacteria).[9,10] Alterations in the normal composition of GCM, known as intestinal dysbiosis, have been linked to several diseases of the GI tract, including inflammatory conditions and IBS.[2,11–16] For instance, in IBS patients, intestinal dysbiosis with altered host-microbial interactions seems to be important generating a local immune response that might lead to the sensorial and secretomotor alterations characteristic of the disease. The underlying mechanisms remain largely unknown, although some evidences support a local modulation of sensory-related systems leading to altered functional responses.[16–19] For instance, we have recently shown that specific alterations in the composition of the GCM modify the expression of the intestinal endocannabinoid system, affecting nociceptive responses in mice.[17]

The intestinal immune system is in the front line of defense against bacteria; tolerating GCM, but, at the same time, maintaining appropriate immune responses to pathogens.[18,20–23] In this context, the innate immune system represents a pivotal player in controlling host resistance

and maintaining the mucosal immune balance. The innate immune system provides a primary host response to bacterial invasion by using pattern recognition receptors (PRRs), mainly Toll-like receptors (TLRs), to recognize microbial agents. TLRs-mediated host-bacterial interactions trigger the sequential activation of intracellular signaling pathways leading to the induction of a range of mediators that drive the primary host resistance to pathogens. Additional innate immune components include the mucous barrier and the secretion of IgA and antimicrobial peptides (AMPs), that modulate luminal microbiota avoiding bacterial attachment to the epithelium.[4,21,22,24]

In the present study, to further understand the role of microbiota influencing gut secretomotor and sensory responses, we assessed changes in the local immune system and in the expression of sensory-related systems within the colon of mice after a 2-week antibiotic treatment-induced dysbiosis. Furthermore, we also assessed if these changes lead to functional alterations displayed as changes in colonic contractility and viscerosensitivity.

Material and Methods

Animals

Female CD1 mice, 10-12 week-old (Charles River Laboratories) were used. All animals were maintained in conventional conditions in an environmentally controlled room (20-22ºC, 12 h light:dark cycle), with food and water *ad libitum*. All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocols 1099 and 1396) and the Generalitat de Catalunya (protocols 5646 and 7193).

Antibiotic treatment

Animals received a mixture of non-absorbable, broad spectrum antibiotics containing Bacitracin A (31626 - Vetranal™; Sigma-Aldrich) and Neomycin (N1876 - Neomycin trisulfate salt hydrate; Sigma-Aldrich). Amphotericin B (A9528; Sigma-Aldrich) was added to prevent yeast overgrowth. During a 2-week period, animals were dosed daily, by oral gavage, with 0.3 mL of the mixture (prepared fresh on a daily basis). This procedure ensured a minimum dose of 0.4 mg for bacitracin and neomycin and 0.1 mg for amphotericin B (per mouse and day). In addition, the same mixture was added to the drinking water during the same period of time. Vehicle-treated

animals were dosed with deionized water by oral gavage (0.3 mL). Water consumption and body weight was assessed on a daily basis during the treatment period. Similar treatment protocols have been followed previously in comparable studies in mice and rats, demonstrating the induction of significant changes of the GCM.[16,17,54]

EXPERIMENTAL PROTOCOLS

Mice were treated with the antibiotic mixture (n=28) or vehicle (n=28) during 14 consecutive days. Different subgroups of vehicle- and antibiotics-treated animals were used to assess visceral pain responses (behavioral pain responses to IP acetic acid, n=22 and IC capsaicin, n=22) and *in vitro* colonic contractility (n=12). On day 15, 24h after the last antibiotic/vehicle administration and after all other procedures (pain tests), animals were euthanized, the weight of body organs assessed and samples of colonic tissue and fecal content obtained. Samples obtained from animals included as controls in the visceral pain tests were used for morphological/molecular studies. Each animal was used only for one procedure.

SAMPLES COLLECTION

Mice were deeply anesthetized with isoflurane (Isoflo) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Thereafter, a medial laparotomy was performed, the ceco-colonic region localized and the cecum and colon dissected. Afterward, ceco-colonic fecal contents and a tissue sample from the proximal colon were collected and frozen immediately in liquid nitrogen. Frozen samples were stored at –80 ºC until analysis. At the same time, tissue samples of the proximal and middle colon (about 1.5 cm each) were collected and fixed overnight in Meta-Carnoy fixative (methanol:chloroform:glacial acetic acid, 6:3:1, v:v:v) or in 4% paraformaldehyde. After an overnight fixing, tissues were paraffin embedded and 5 µm-tick sections obtained. During the necropsy, the adrenal glands, the thymus, the liver and the spleen were dissected and weighed.

QUANTIFICATION OF BACTERIA USING REAL-TIME QUANTITATIVE PCR (QPCR)

Total DNA was isolated from frozen fecal ceco-colonic content using QIAamp® DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions. Thereafter, DNA was quantified using

the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Fisher Scientific Spain, Madrid, Spain), diluted to equal concentrations with sterile deionized water and stored at -20ºC until analysis.

The relative abundance of bacteria was measured using 16S rRNA gene-targeting hydrolysis probes (Custom TaqMan assays; Applied Biosystems) as previously described.[55–57] Probes used are detailed in Table 1. All samples and the negative controls were assayed in triplicates. The vehicle group served as the calibrator. For each assay, a positive (quantified) sample was used to generate a standard curve and to quantify the number of bacteria. For Clostridia cluster XIVa and total bacterial counts, *C. coccoides* (DSM 935; German Collection of Microorganisms and Cell Cultures) was used. In all cases, standard curves were derived from the serial dilutions in a customary way [EUB (r^2=0.99): y = -0.2808x + 16.023; EREC (r^2=0.99): y = -0.2964x + 16.749 ; LAB (r^2=0.98): y = -0.3342x + 18.513; BIF (r^2=0.99): y = -0.2777x + 12.908; and BAC (r^2=0.99): y = -0.2906x + 13.935]. Relative concentrations were expressed in arbitrary units. Logarithms (base 10) of concentrations were plotted against crossing points and Least square fit was used as the standard curve to obtain the bacterial number (in cells ml^{-1}).

IDENTIFICATION OF BACTERIAL ADHERENCE BY FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Fluorescence *in situ* hybridization (FISH) procedures for colonic tissue samples were followed as previously reported.[17,35] Oligonucleotide probes consisted in a single strain DNA covalently linked with a Cy3 (carbocyanine) reactive fluorescent dye at the 5' end (Biomers and Isogen Lifescience). Probes used are detailed in Table 1.

Hybridized slides were viewed under oil immersion, using a Carl Zeiss Axioskop 40 FL epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (Zeiss AxioCam MRm) for obtaining digital images (Zeiss AxioVision Release 4.8.1). 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 15% of the pictures observed (at least 3 out of 20) was required to decide that there was bacterial attachment to the epithelium. All procedures were performed on coded slides, to avoid bias.

Table 1. Probes and primers used for bacterial identification (FISH and qPCR).

	FISH (5' – 3') [1]	Ref.		qPCR (5' – 3')	Ref.
Non bacteria NON 338	ACATCCTACGGGAGGC	17,60			
Total bacteria EUB 338	GCTGCCTCCCGTAGGAGT	17,60	F[2]	CGGTGAATACGTTCCCGG	56
			R[3]	ACGGCTACCTTGTTACGACTT	
			P[4]	GTACACACCGCCCGTC	
Enterobacteria ENT-D	TGCTCTCGCGAGGTCGCTT CTCTT	17,60			
***Bacteroides* spp** BAC 303	CAATGTGGGGGACCTT	17,60	F	AGAGGAAGGTCCCC	57
			R	GCTACTTGGCTGGTT	
			P	CATTGACCAATATTCCTCACTG CTGC	
***Bifidobacterium* spp** BIF 164	CATCCGGCATTACCACCC	17,60	F	CGTGCTTAACACATGCAA	57
			R	CACCCGTTTCCAGGAG	
			P	TCACGCATTACTCACCCGTTCG	
***C. coccoides* (cluster XIVa)** EREC 482	GCTTCTTAGTCAGGTACCG	17,60	F	GACGCCGCGTGAAGGA	56
			R	AGCCCCAGCCTTTCACATC	
			P	CGGTACCTGACTAAGAAG	
***Lactobacillus-Enterococccus* spp** LAB 158	GGTATTAGCACCTGTTTCCA	17,60	F	TGGATGCCTTGGCACTAGGA	61
			R	AAATCTCCGGATCAAAGCTTA CTTAT	
			P	TATTAGTTCCGTCCTTCATC	
Verrucobacteria MUC1437	ATGTGCCGTCCGCGGGTT	62			
Segmented filamentous bacteria SFB 1008	GCGAGCTTCCCTCATTACAA GG	63			

[1]: Probe (Cy3-5' – 3'); [2]:Forward primer; [3]:Reverse primer; [4]: Probe (FAM-5' – 3').

QUANTIFICATION OF SECRETORY IMMUNOGLOBULIN A

Luminal s-IgA was measured in fresh homogenates of cecal contents (equal diluted in PBS 1x) using a commercial double-antibody sandwich ELISA, following manufacturers' instructions (MBS564073; MyBiosource).

MRNA ANALYSIS

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit (Ambion/Applied biosystems) using the FastPrep-12 instrument (MP Biomedicals, France). Thereafter, a two-step quantitative real time PCR (RT-qPCR) was performed. cDNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems , Foster City, CA). The PCR reaction mixture was incubated on the ABI 7500Fast (Applied Biosystems). All samples, as well as the negative controls, were assayed in triplicates. The cycle thresholds for each sample were obtained and data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the vehicle group serving as the calibrator.[58]

TaqMan ® gene expressions assays (hydrolysis probes*)* for cannabinoid receptor 1 (CB1) (Mm01212171_s1) and 2 (CB2) (Mm00438286_m1), mu-opioid receptor (MOR) (Mm01188089_m1), tryptophan hydroxylase 1 (TPH1) (Mm00493794_m1) and 2 (TPH2) (Mm00557715_m1), serotonin Transporter (SERT) (Mm00439391_m1), transient Receptor Potential Vanilloid 1 (TRPV1) (Mm01246302_m1) and 3 (TRPV3) (Mm00455003_m1), nerve growth factor (NGF) (Mm00443039_m1), interleukin 6 (IL-6) (Mm00446190_m1), tumor necrosis factor α (TNFα) (m00443258_m1), interleukin 12 (IL-12p40) (Mm00434174_m1), interleukin 10 (IL-10) (Mm00439614_m1), toll-like receptor (TLR) 2 (Mm00442346_m1), 3 (Mm01207404_m1), 4 (Mm00445273_m1), 5 (Mm00546288_s1) and 7 (Mm00446590_m1), defensin-α6/24 (Mm04205950_gH), defensing-β4 (Mm00731768_m1), the lectin regenerating islet-derived protein 3 gamma (RegIIIγ) (Mm00441127_m1) and the resistin-like molecule-β (RELMβ) (Mm00445845_m1) were used (Applied Biosystems). β-2-microglobulin (Mm00437762_m1) was used as endogenous reference gene.

HISTOLOGY

For histological examination, hematoxylin-eosin-stained sections from the colon were obtained following standard procedures. 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.

The mucous layer was assessed in Meta-Carnoy-fixed samples of colonic tissue. Thickness of the mucous layer was measured in 10 different fields, in representative regions covering, at least, 20% of the epithelial surface. All measurements were performed on coded slides by two independent investigators using the Zeiss AxioVision Release 4.8.1 software. Moreover, tissue sections were also stained with Alcian Blue pH 2.5/Periodic Acid Schiff (AB 2.5/PAS kit; Bio-Optica) in order to specifically stain neutral (pink), mixed (purple) or acidic (blue) mucins. Thereafter, colonic goblet cells were counted in 20 longitudinally-oriented villus-crypt units. Length of the villus-crypt unit was also determined to obtain goblet cells density (number of cells mm^{-1}).

Immunohistochemistry and quantification of Immune-Positive Signal in the Myenteric Plexus

Paraffin-embedded tissue sections (5 μm in thickness) were deparaffinized and rehydrated with a battery gradient of alcohols. Immunohistochemistry protocols for each antibody were followed by a customary way and as previously described.[55]Antigen retrieval for CB1 receptor and MOR was achieved by processing the slides in a microwave with 10 mM of citrate solution. Epitope retrieval for CB2 receptor was performed using a pressure cooker (at full pressure, for 3 min) in Tris–EDTA solution buffer. Primary antibodies included a rabbit polyclonal anti-CB1 (1:100; rabbit polyclonal to cannabinoid receptor 1, ab23703; Abcam), a rabbit polyclonal anti-CB2 [1:100; rabbit polyclonal to cannabinoid receptor 2 (H-60), sc-25494; Santa Cruz Biotechnology], and a rabbit polyclonal anti-MOR (1:2,500; rabbit polyclonal to mu-opioid receptor AB1580; Chemicon/Millipore). The secondary antibody used was a biotinylated polyclonal swine anti-rabbit IgG (E 0353; DakoCytomation). Antigen–antibody complexes were reveled with 3-3'-diaminobenzidine (SK-4100 DAB; Vector Laboratories), with the same time exposure per antibody,

and sections were counterstained with hematoxylin. Specificity of staining was confirmed by omission of the primary antibody.

For each marker assessed (CB1, CB2 and MOR), immunopositive cells were counted in 30, randomly selected, myenteric ganglia for each tissue sample. Cells were considered to be immunopositive if they expressed more labeling than the background levels seen in the negative controls. Myenteric ganglia of the same animals were photographed (Eclipse 90i, Nikon), and the area quantified (in μm^2) using the software ImageJ (NIH, National Institutes of Health, USA). All procedures were performed on coded slides to avoid any bias.

ORGAN BATH CONTRACTILITY OF THE COLON

Full thickness preparations from the mid portion of the colon were cut 0.2 cm wide and hung for organ bath study oriented to record circular muscle activity. Strips were mounted under 1-2 g tension in a 10 mL organ bath containing carbogenated (95% O2–5% CO2) Krebs solution with glucose and maintained at 37±1ºC. One strip edge was tied to the bottom of the organ bath using 2/0 silk suture and the other one to an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA). Output from the transducer was fed to a PC through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain). Strips were allowed to equilibrate for about 90 min; thereafter, to determine the spontaneous contractile activity, the tone was measured for 10 min. After this, responses to carbachol (CCh; 10^{-7} to 10^{-4}M; Sigma-Aldrich), added to the bath in a cumulative manner at 5-min intervals, were assessed. Thereafter, the bath solution was replaced, tissues were allowed to reequilibrate (20 min), and spontaneous contractile activity and responses to the NO-synthase inhibitor NG-nitro-L-arginine (L-NNA; 10^{-3}M; Sigma-Aldrich) were assessed. The amplitude of contractions from the baseline (maximal response) and the area under curve (AUC) during 10 min (spontaneous activity and effects of L-NNA) or 5 min (effects of CCh) were used to evaluate the contractile activity.

BEHAVIORAL PAIN RESPONSES TO INTRAPERITONEAL ACETIC ACID

0.6% glacial acetic acid (Sigma-Aldrich) in distilled water (10 ml/kg) or vehicle (distilled water) was administered and pain related responses were determined following previous protocols.[59] The pain response was scored by counting the number of abdominal contractions

during the 30 min period after the IP treatment (in blocks of 5 min) by two independent researchers.

Behavioral responses to Intracolonic capsaicin-evoked visceral pain

Spontaneous visceral pain-related behaviors induced by intracolonic capsaicin (Sigma-Aldrich) were assessed following previously described protocols, with minor modifications.[17] Mice were anesthetized with isoflurane (Isoflo; Esteve) and capsaicin (0.05 ml/mice, 0.1% in ethanol:Tween 80:saline; 1:1:8, v:v:v; Sigma-Aldrich) or vehicle (ethanol:Tween 80:saline; 1:1:8, v:v:v) were administered intracolonically. Petroleum jelly was applied to the perianal area to avoid the stimulation of somatic areas due to any leakage on the capsaicin solution. After recovering consciousness, visceral pain-related behaviors (licking of the abdomen, stretching the abdomen, squashing of the lower abdomen against the floor or abdominal retractions) were assessed during a 30 min period (in blocks of 5 min). Pain behaviors were visually assessed by two independent researchers.

Statistical analysis

Data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Data were analyzed by a parametric unpaired t-test or by a non-parametric Mann-Whitney test as appropriate. A Chi-square test was used to analyze bacterial adherence. Data were considered statistically significant when $P<0.05$.

Results

Clinical and macroscopical assessment of the animals

During the 2-week antibiotic treatment, no clinical signs were observed, with all animals showing a similar rate of body weight gain (data not shown). Water intake was similar across experimental groups (data not shown). At necropsy, the only significant change observed was the enlargement of the cecum in antibiotic-treated animals (507.7±18.43 mg, $P<0.0009$ vs. vehicle group: 409.0±20.24 mg; n=19 for each). These differences were maintained when the cecal weight was expressed as relative to the total body weight (data not shown).

HISTOLOGICAL EVALUATION

Microscopic analysis of colonic tissue samples showed a normal histological structure in all animals. Occasionally, a moderate multifocal-to-diffuse inflammatory infiltrate and/or desquamation of some epithelial cells were observed, but no treatment-related incidence could be established. No significantly differences in the final histopathological scores (vehicle: 0.66±0.25; antibiotic: 1.27±0.25; n=7-8 per group) were observed (Fig. 1A).

Evaluation of PAS/AB-stained sections showed a reduction in the density of goblet cells (per crypt length) in antibiotic-treated animals (158.8±8.7 cells mm^{-1}, n=8) compared with the vehicle-treated group (187.2±7.4 cells mm^{-1}, n=7, P<0.05; Fig. 1B). This was associated to a tendency for colonic crypts length to be increased in antibiotic–treated mice (vehicle: 87.52±4.9 µm; antibiotic: 101.9±4.97 µm; P=0.061; Fig. 1C). When differentiating between acidic, mixed or neutral mucins, antibiotic-treated mice showed a relative increase in the number of goblet cells containing a mixture of acidic and neutral mucins (antibiotic: 21.1±1. 9 cells mm^{-1}; vehicle: 28.5±2.0 cells mm^{-1}; P<0.05; Fig. 1B). No differences were observed for the thickness of the mucus layer (Fig. 1D).

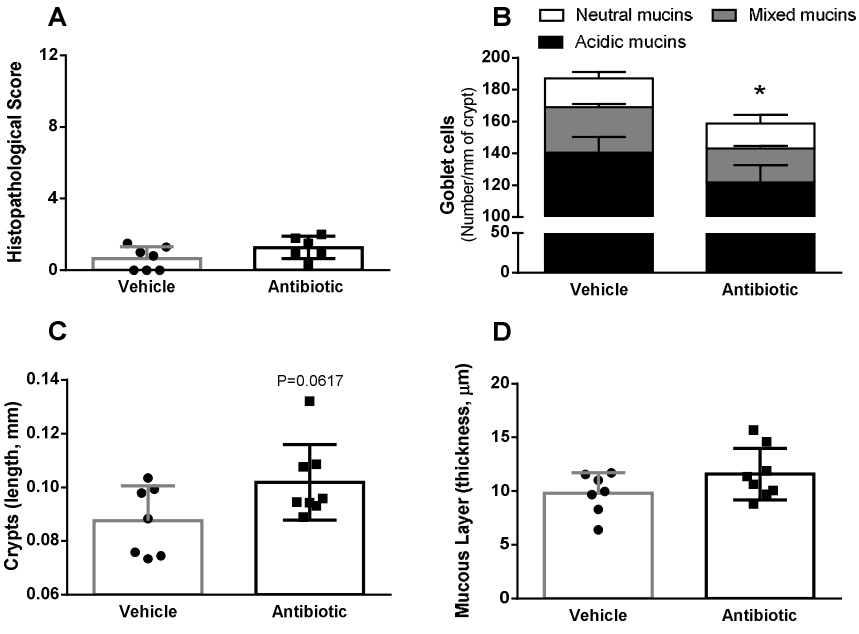


Figure 1. Colonic histopathology in vehicle- and antibiotic-treated mice. A: Histopathological scores. B: Goblet cell counts from PAS/AB pH=2.5 stained-sections. C: length of colonic crypts. D: Thickness of the mucus layer, assessed on PAS/AB pH=2.5 stained-sections. Bars represent the mean±SEM, symbols represent individual animals. n=7-8 per group, *: P<0.05 vs. vehicle.

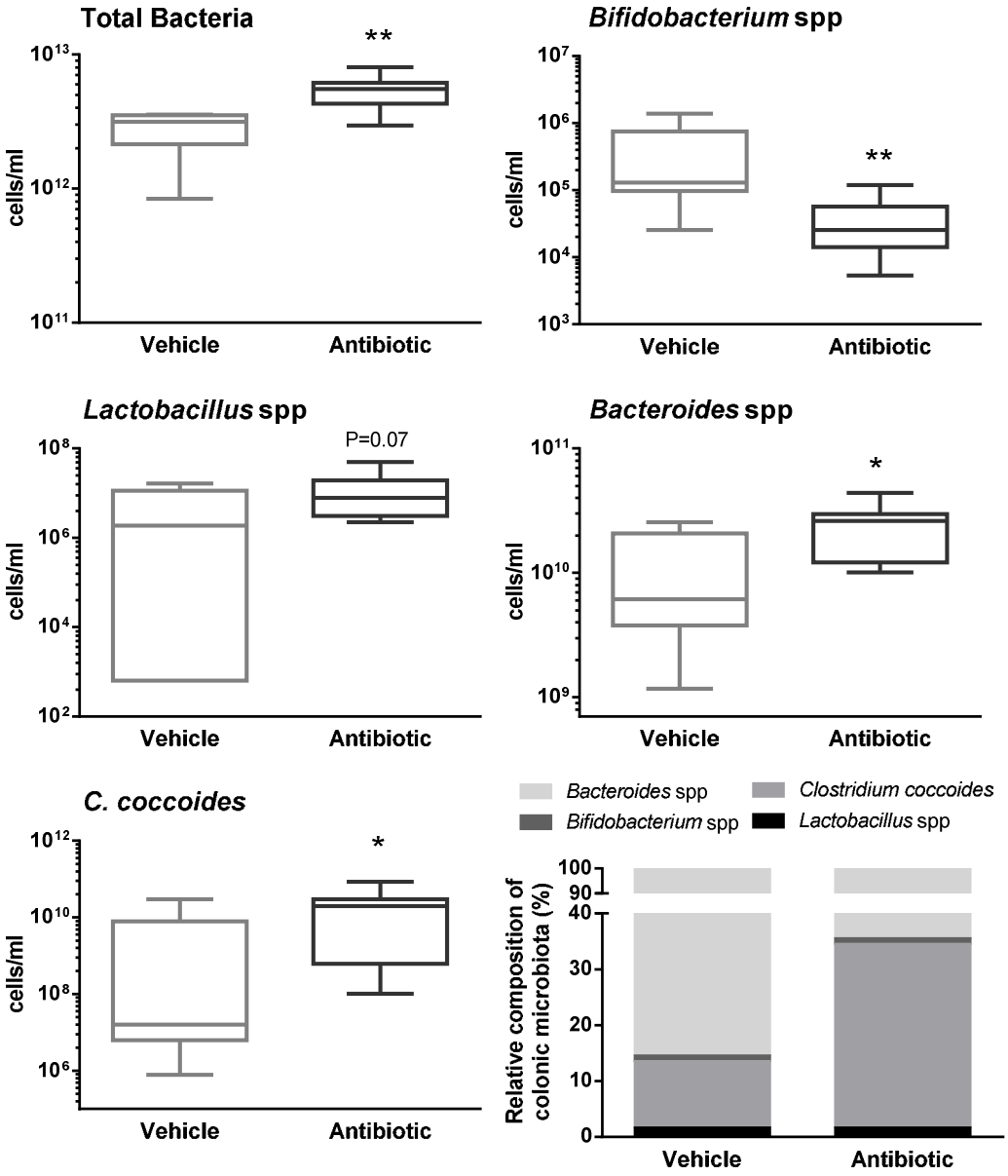


Figure 2. Characterization and quantification of luminal gut commensal microbiota. Data shows qPCR quantification of total bacteria and the main bacterial groups present in the colonic microbiota (see methods for details). Data are median (interquartile range) ± SD; n=7-8 for each group. The bottom-right graph shows the relative distribution of the ceco-colonic microbiota in vehicle- and antibiotic-treated mice. Data represent the relative abundance (percent) of the main bacterial groups present in the gut microbiota as quantified using qPCR. Relative percent composition was calculated taking as 100% the total counts of the different bacterial groups assessed (*C. coccoides*, *Bacteroides* spp., *Bifidobacterium* spp. and *Lactobacillus*/*Enterococcus* spp.).

A 2-WEEK ANTIBIOTIC TREATMENT RESULTS IN A DYSBIOSIS OF THE COLONIC COMMENSAL MICROBIOTA

Total bacterial counts were increased in antibiotic-treated mice for a 2-week period when compared with the counts in vehicle-treated animals (5.33±0.54 x 10^{12} cells ml^{-1}vs. 2.79±0.38 x 10^{12} cells ml^{-1}; P=0.004;). The treatment with antibiotics increased *Bacteroides* spp. and *Clostridium coccoides* counts by 2- and 4-fold, respectively (both P<0.05; Fig. 2). Similarly, the Lactobacilli group was increased by 3-fold after the antibiotic treatment although statistical significance was not reached, probably because of the relative large variability observed in control conditions (P=0.07; Fig. 2). On the other hand, the Bifidobacteria group showed a 10-fold reduction in antibiotic-treated animals (P=0.006; Fig. 2).

When assessing the relative composition of the microbiota (proportion of each bacterial group assessed within the total counts), the most abundant bacterial characterized, regardless of the treatment, were *Bacteroides* spp. and *C. coccoides (*cluster XIVa), representing 99% of the total bacterial counts. During antibiotic treatment the main change was an increase in the ratio of *C. coccoides (*cluster XIVa), from about 15% in control conditions to approximately 35% of the total counts. Regardless the treatment, the lactobacilli group was very scarce (<0.05% of the total bacterial counts) and the Bifidobacteria group was the less abundant (<0.01% of the total bacterial counts) (Fig. 2).

Table 2. Incidence of bacterial wall adherence[1].

	***Bacteroides* spp.**	**Enterobacteria**	**Verrucobacteria**	***Clostridium* cluster XIVa**	***Lactobacillus-Enterococcus* spp.**	***Bifidobacterium* spp.**	**SFB**
Vehicle	1/8 (12.5%)	3/8 (37.5%)	0/8 (0%)	8/8 (100%)	1/8 (12.5%)	0/8 (0%)	2/8 (25%)
Antibiotic	0/7 (0%)	2/7 (28.6%)	0/7 (0%)	6/7 (85.7%)	1/7 (14.3%)	1/7 (14.3%)	2/7 (28.6%)

[1]: Data represent the number of animals showing bacterial wall adherence over the total of animals assessed (percentage of incidence). SFB: segmented filamentous bacteria.

In control conditions, the main bacterial group adhered to the colonic epithelium was Clostridia (incidence of attachment: 100%), followed by Enterobacteria (incidence of attachment: 38%) and Segmented Filamentous Bacteria (Incidence of attachment: 25%). Overall, the 2-week period of antibiotic treatment did not affect the ratios of bacterial wall adherence (Fig. 3, Table 2). Nevertheless, FISH images revealed that antibiotic-treated mice showed a higher proportion of

coccoid-shaped Clostridia adhered to the epithelium than vehicle-treated animals, in which Clostridia hybridized mainly as a fusiform ballici (Fig. 3).

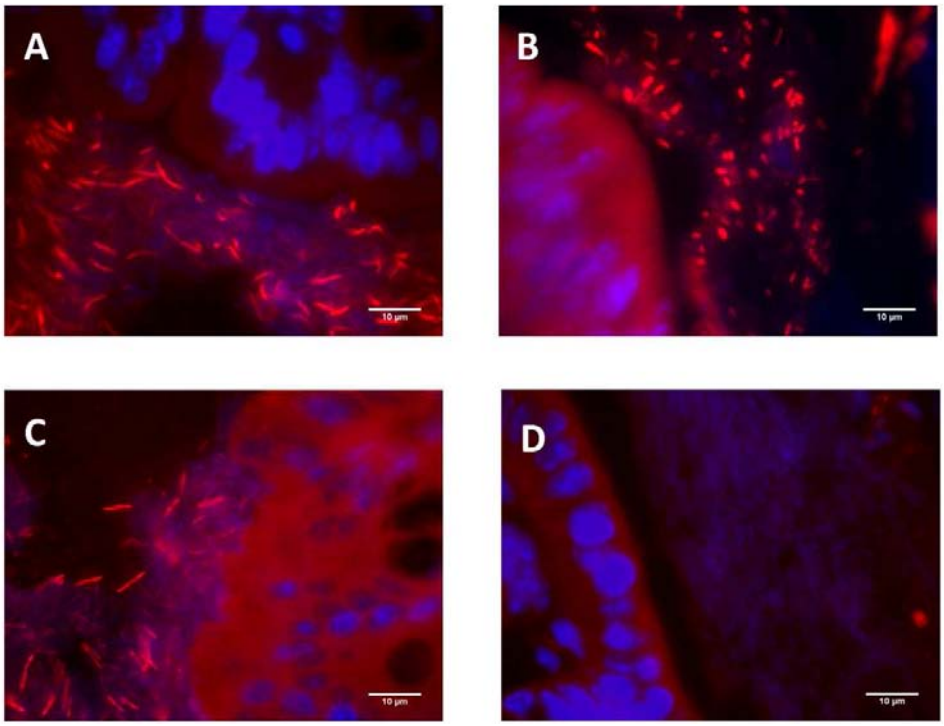


Figure 3. Representative colonic tissue images showing Clostridium spp. (identified by FISH using the EREC 482 probe) adherence to the colonic epithelium. A: Vehicle-treated animal. B: Antibiotic-treated animal. C: Non-treated naïve animal maintained in the same conditions as the experimental groups; included here for comparative purposes. D: Negative control (hybridized with the control non-specific fluorescent probe NON338). In all cases (A-C) abundant bacteria was observed attached to the colonic epithelium. Note, however, that bacillary-shape bacteria were observed in vehicle-treated animals (A) (similarly to that observed in the non-treated naïve animal, C) while in antibiotic-treated animals (B) a shift in morphology, with the appearance of abundant coccoidal forms, can be observed.

ANTIBIOTICS MODULATED THE LOCAL INNATE IMMUNE SYSTEM

S-IgAs were detected in all luminal contents analyzed. Levels of s-IgA were increased by 10-fold in antibiotic-treated animals ($P<0.05$, Fig. 4A).

A relatively high variability was observed in the expression levels or AMPs. In control conditions, the relative expression levels of AMPs were: RELMβ > RegIIIγ > defensin-α24/6; with defensin-β4 being undetectable in all samples analyzed. In antibiotic-treated mice, RELMβ was down-regulated (P<0.05 vs. vehicle group) while RegIIIγ tended to be up-regulated, although without reaching statistical significance (Fig. 4A).

No differences in the expression of pro- (IL-6, IL-12p40 and TNFα) or anti-inflammatory (IL-10) cytokines was observed between vehicle- and antibiotic-treated animals. Only IL-12p40 tended to be up-regulated in antibiotic-treated mice (P=0.069, Fig. 4B).

In control conditions, relative colonic expression of TLRs was: TLR4 > TLR3 > TLR2 > TLR5 > TLR7. In antibiotic-treated animals specific changes in expression were detected; with a significant up-regulation of TLR7 (P=0.008) and a tendency for TLR4 (P=0.08); while TLR5 showed a slight (less than one fold) down-regulation (P=0.02) (Fig. 4C). Expression of other TLRs was not affected.

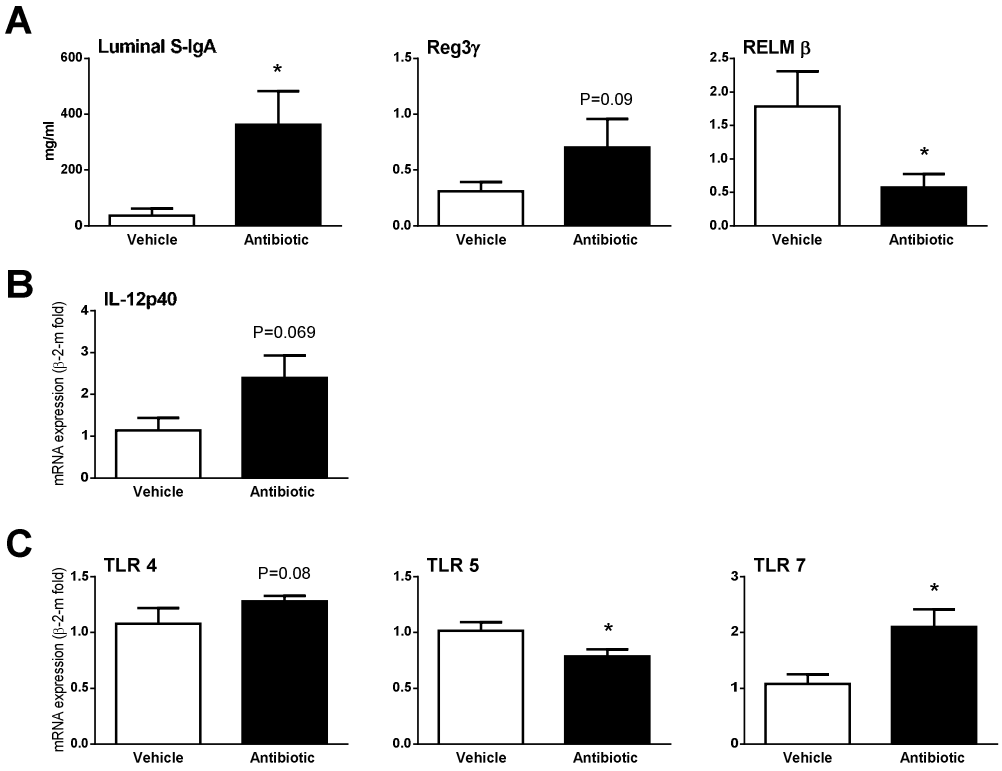


Figure 4. Changes in immune and host-bacterial interaction markers. A: Changes in innate immune-related markers: luminal levels of secretory IgA (S-IgA) and gene expression levels of antimicrobial peptides. B: Changes in expression levels of IL-12p40. C: Changes in the expression levels of TLRs. Data are mean±SEM, n=7-8 group, *: P<0.05 vs. vehicle.

ANTIBIOTICS MODULATED THE LOCAL EXPRESSION OF SENSORY-RELATED MARKERS

With the exception of tryptophan hydroxylase 2 (which was in general undetectable) all markers assessed were expressed at detectable levels in all samples analyzed. In antibiotic-treated animals, only a selective down-regulation of CB1, MOR and NGF was detected (Fig. 5A). CB2 expression showed a tendency to be up-regulated; however, statistical significance was not reached probably because of the relatively high variability observed in antibiotic-treated animals. Other secretomotor and sensory markers assessed were not affected by the antibiotic treatment.

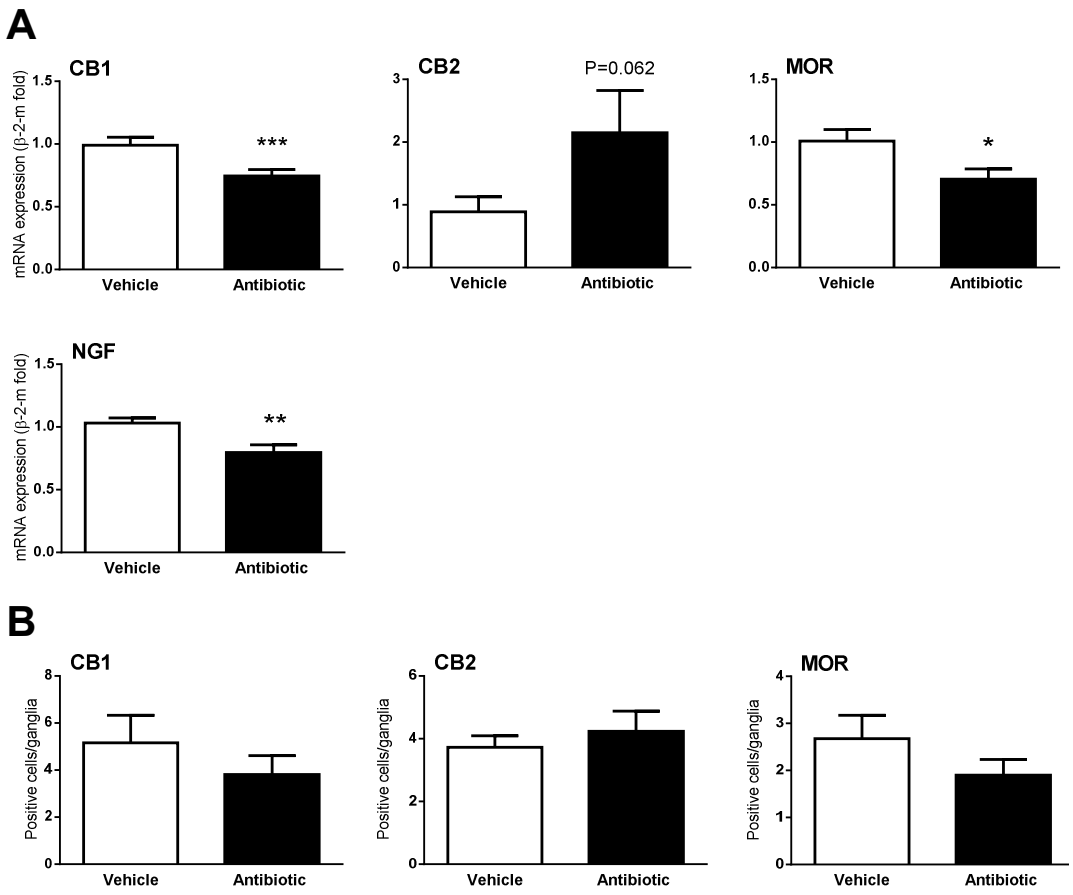


Figure 5. Changes in sensory-related markers. A: Changes in colonic gene expression of cannabinoid receptors 1 and 2 (CB1/2), mu-opioid receptors (MOR) and nerve growth factor (NGF). Data are mean±SEM, n=5-8 animals per group. *, **, ***: P<0.05, 0.01 or 0.001 vs. vehicle. B: Quantification of immunorreactive ganglionic cells within the myenteric plexus in vehicle- and antibiotic-treated animals. Data are mean±SEM, of 5-8 animals per group; see methods for details of the quantification procedures.

Evaluation of CB1, CB2 and MOR content in the colonic myenteric plexus using immunohistochemistry correlated with the gene expression data, although statistical significances were not reached. The number of CB1- or MOR-positive ganglionic cells within the myenteric plexus was reduced by 25% and 30%, respectively, in antibiotics-treated mice. On the other hand, the number of CB2-positive ganglionic cells was increased by 13% in antibiotic-treated animals (Fig. 5B). The mean area of the myenteric ganglia was similar in vehicle- and antibiotic-treated mice (vehicle: 57.1±10.1 μm^2, antibiotic: 71.0±19.0 μm^2, P>0.05).

BACTERIAL COUNTS CORRELATED WITH HOST-BACTERIAL INTERACTION AND NOCICEPTIVE MARKERS

Significant correlations were found between bacterial counts and the expression changes in host-bacterial interaction and nociceptive markers. Total bacterial counts correlated negatively with the colonic expression of CB1 (P=0.01; r^2=0.39) and TLR-5 (P=0.02; r^2=0.36) and positively with CB2 (P=0.03; r^2=0.31) and TLR-7 (P=0.02; r^2=0.32) (Fig. 6A).

Moreover, regardless the treatment applied, positive correlations between TLR7 and the nociceptive markers CB2 (P=0.0001; r^2=0.79), TRPV1 (P=0.005; r^2=0.45) and TRPV3 (P=0.0008; r^2=0.59) were found, while negatively correlating with MOR expression (P=0.01; r^2=0.39) (Fig. 6B). In addition, expression levels of IL-12p40 (P=0.0008; r^2=0.59) and IL-10 (P=0.003; r^2=0.49) correlated positively with TLR7 expression.

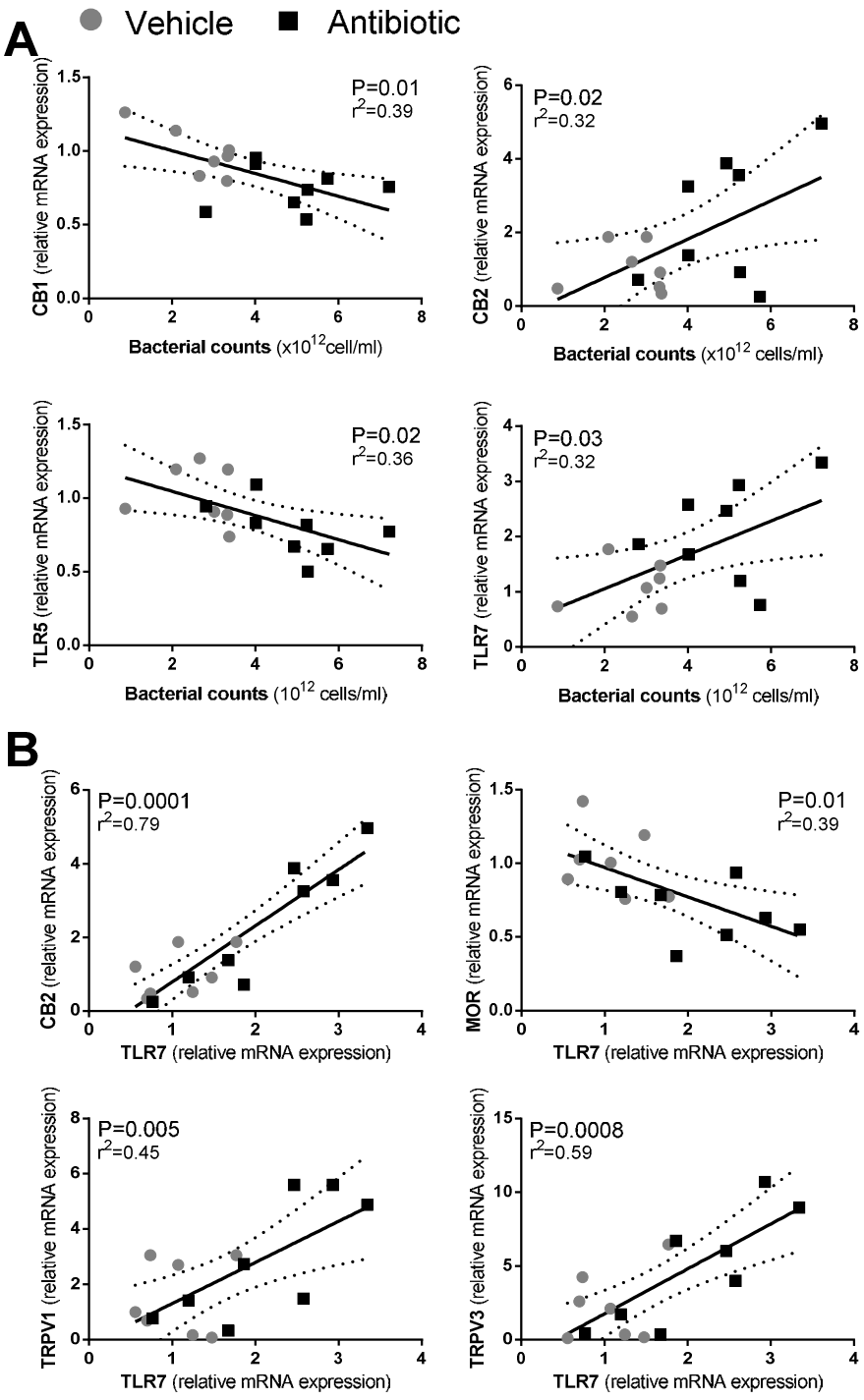


Figure 6. A: Correlations between total luminal bacterial counts and sensory-related (CB1 and CB2) markers or TLRs. B: Correlations between expression levels of TLR7 and sensory-related markers. Each point represents an individual animal. Broken lines represent the 95 % confidence interval.

VISCERAL PAIN-RELATED RESPONSES WERE ALTERED IN ANTIBIOTIC-TREATED MICE

Intraperitoneal acetic acid produced repeated characteristic stretching contractions (abdominal contractions) during the 30 min period after administration, with a maximal response observed at 10 min post-administration. Time-course responses to IP acetic acid were similar in vehicle- and antibiotic-treated mice, but the overall response was attenuated by 33% in antibiotics-treated mice (40.9±7.6 abdominal contractions/30 min, n=6) when compared to vehicle treated animals (61.4±4.0 abdominal contractions/30 min, n=6; P<0.001; Fig. 7A). Abdominal contractions were absent in animals injected IP with vehicle.

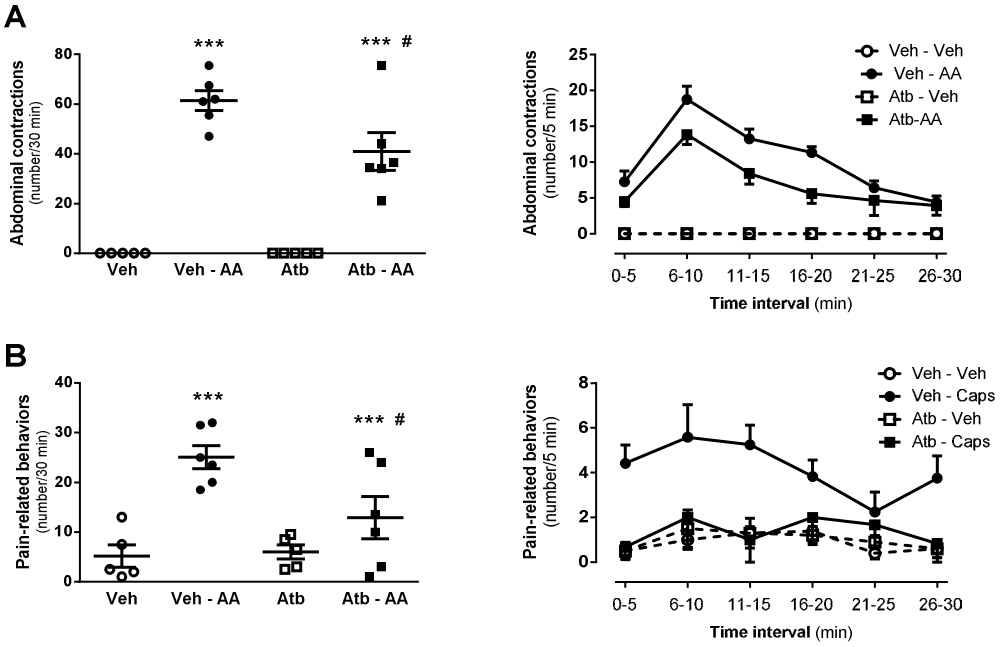


Figure 7. Effects of antibiotic treatment on visceral pain-related responses. A: Intraperitoneal acetic acid- (AA, 0.6%) induced abdominal contractions. The left graph shows the total number of abdominal contractions during the observation time (30 min) in the different experimental groups (Veh- vehicle and Atb-antibiotic). Each point represents an individual animal; the horizontal lines with errors correspond to the mean±SEM. ***: P<0.001 vs. respective non-AA-treated control group. #: P<0.05 vs. vehicle-AA group. The graph to the right shows the time-course (in 5-min intervals) for the pain-related responses in the same animals. B: Intracolonic capsaicin- (Caps) evoked visceral pain-related behaviors. The left graph shows the total number of behaviors during the observation time (30 min) in the different experimental groups. Each point represents an individual animal; the horizontal lines with errors correspond to the mean±SEM. ***: P<0.001 vs. respective non-capsaicin-treated control group. #: P<0.05 vs. vehicle-Caps group. The graph to the right shows the time-course (in 5-min intervals) for the observation of pain-related behaviors in the same animals.

Intracolonic administration of capsaicin induced pain-related behaviors in all mice during the 30 min observation period, with a maximal response observed at 10 min post-administration. Capsaicin-induced pain-related behaviors were reduced by 48% in antibiotic-treated mice (12.9±4.2 behaviors/30 min, n=6) when compared to vehicle-treated mice (25.1±2.3 behaviors/30 min, n=6; $P<0.05$; Fig. 7B). The predominant behavior after intracolonic capsaicin was the licking of the abdominal area, observed in all animals. Stretching of the abdomen and squashing of the lower abdomen against the floor behaviors, considered to reflex the highest levels of pain, were seen only in vehicle-treated mice.

COLONIC CONTRACTILITY WAS ALTERED IN ANTIBIOTIC-TREATED MICE

Spontaneous colonic contractility, as assessed *in vitro*, was increased in the antibiotic-treated group (AUC/10 min: 2.73±0.46 g, n=6) when compared to vehicle-treated animals (AUC/10 min: 1.60±0.14 g, n=5; $P<0.05$; Fig. 8). Regardless the experimental group considered, carbachol produced a concentration-dependent contractile response. In antibiotic-treated mice the EC_{50} for carbachol was 2.4-fold lower than that determined in control conditions (vehicle: 1.24±0.30 10^{-6} M; antibiotic: 5.19±1.59 10^{-7} M; n=5-6; P=0.056; Fig. 8).

Spontaneous colonic contractility during L-NNA addition to the organ bath, to block NO synthesis, had a tendency to be higher in antibiotic-treated mice (AUC/10 min: 10.37±2.77 g, n=6) compared with vehicle controls (AUC: 4.88±0.82 g, n=5; P=0.0578; Fig. 8).

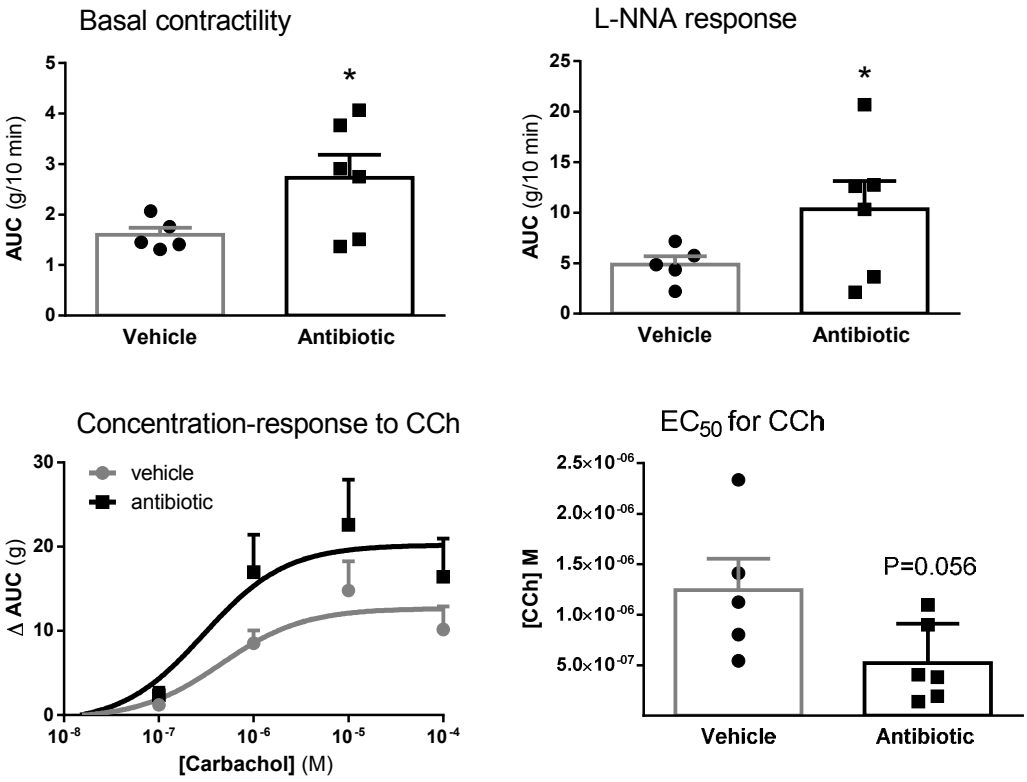


Figure 8. Effects of antibiotic treatment on colonic contractility assessed in vitro: basal contractility; contractile responses to NO-synthase inhibition with LNNA; Concentration-response curves to cholinergic stimulation with carbachol (CCh) and corresponding EC50s. Data are mean±SEM, n=5-6 per group, each point represents an individual animal (except for the concentration-response curves, where only mean±SEM is shown). *: P<0.05 vs. vehicle.

DISCUSSION

In the present study, we show that a 2-week treatment with antibiotics generates a moderate dysbiotic state, with increments in total bacterial counts, in mice. These changes imply a modulation of both host-bacterial interaction systems and local neuro-immune systems; leading to functional alterations revealed as changes in colonic sensitivity and motor activity.

The 2-week antibiotic treatment caused a specific dysbiosis accompanied with an enlargement of the cecum, although no signs of inflammation were observed, as previously described.[17,25,26] Overall, this dysbiotic state is similar to that described in previous studies in mice and indicates antibiotic-induced temporal and spatial changes in the GCM composition.[17,25,27] In the present conditions, total bacterial load was increased in antibiotic-treated animals, suggesting

that the treatment favored the expansion of antibiotic-resistant bacterial groups. This contrasts with other studies indicating that antibiotics alter bacterial community richness decreasing overall bacterial density.[24–26] In the same way, similar antibiotic treatment might cause different microbial changes depending upon the doses administered, the basal microbial composition, the strain and commercial breeder of the animals used or the environmental conditions. Specifically, short- and long-term antibiotic treatments seem to generate different states of dysbiosis[25,27]which can be related to different responses of the host.

In our conditions, luminal ceco-colonic dysbiosis was characterized by increments in *Bacteroides* spp, *C. coccoides* (cluster XIVa) and *Lactobacillus-Enterococcus* spp. and reductions in *Bifidobacterium* spp. counts. All these bacteria have been implicated in both GI physiology and pathology.[16,19,24,28–30] Recent evidences suggest a key role of some bacteria in immune functions (mainly segmented filamentous bacteria, SFB, and Clostridia from the clusters XIVa and VI) which are, in normal conditions, in direct contact to the host and, therefore, directly influencing host immune responses.[17,31–33] In agreement with this, we found a large proportion of Clostridia, SFB, and Enterobacteria adhered to the colonic epithelium. However, despite the luminal dysbiosis observed, antibiotics did not modify the rate of attachment, in agreement to that previously reported during long-term antibiotic treatment in mice.[26] Nevertheless, these results contrast with data obtained during short-term antibiotic treatment, which suggested a facilitation in bacterial attachment.[17] These differences further enfatize the importance of the duration of the antibitotic exposure in the microbial and functional responses elicited. Interestingly, a change in bacterial morphology for epithelium-attached Clostridia was observed in antibiotic-treated animals. While in control conditions Clostridia showed a predominant bacillary shape after antibiotics treatment, a higher proportion of coccoidal Clostridia was observed in close contact to the colonic epithelium. This agrees with the key role given to *C. coccoides* in immune activation within the gut.[31] Together with the increase in the ratio of luminal *Clostridium* spp. observed in antibiotic-treated animals, these observations further suggest that antibiotics favored the proliferation of some bacterial groups, particularly *C. coccoides*.

In antibiotic-treated mice, changes in markers related to host-bacterial interactions were detected. TLRs are primary sensors of luminal bacteria and key components in hos-bacterial interactions. In our conditions, there was a type-specific modulation of TLRs expression, with an up-regulation of TLR4 and 7 and a down-regulation of TLR5. Changes in TLR5 and TLR7 might be

difficult to correlate with specific microbial changes because they recognize components of a wide variety of ligands from both Gram positive and negative bacteria.[34] On the other hand, TLR4 is mainly activated by lipopolysaccharides (LPS) from Gram negative bacteria.[34] Therefore, in our conditions, the slight TLR4 up-regulation observed might represent and adaptive response to the proliferation of *Bacteroides* spp. In fact, similar up-regulation of TLR4 was observed in estates of colitis characterized by increases in the counts of gram-negative bacteria (*Bacteroides* spp.)[35] and, on the contrary, a TLR4 down-regulation was detected when *Bacteroides* spp. counts were reduced.[36]

Other markers assessed indicate more extended changes in the local innate immune system. These included changes in the mucosal barrier, the secretion of sIgA and the up-regulation of AMPs and pro-inflammatory cytokines. Overall, these changes indicate an activation of the innate immune system, which might be related to the dysbiotic state. Antibiotic-treated mice showed similar morphological (cecal enlargement, changes in goblet cells) and innate immune-related responses as those observed in other models of antibiotic-induced dysbiosis, in germ free animals or in different models of intestinal inflammation.[25,37,38] These changes might represent a general adaptive pattern of the host in response to alterations in the composition of the microbiota and, therefore, in host-bacterial interactions.

In addition, markers involved in colonic sensitivity and secretomotor responses were also affected during the antibiotic treatment, thus indicating that local adaptive processes to microbial modifications might take place at multiple levels and affect various regulatory systems. From the markers assessed, CB1 and MOR showed a down-regulation while CB2 had a tendency to be up-regulated. Similarly to that observed here, during spontaneous adaptive changes of the microbiota or during the administration of probiotics, both the endocannabinoid and opioid systems were modulated.[17,19,39,40] However, the changes observed contrast with those after short (1-week) treatment with the same antibiotic regime, in which CB2 was up-regulated, without changes in CB1 expression.[17] These differences are likely to relate to the different duration of the antibiotic treatment, reflecting time-related variations (1 wk vs. 2 wk) in the adaptive process of sensory mechanisms. In any case, these observations indicate that modulation of cannabinoid and opioid pathways might be important in host-bacterial interactions and might mediate neural-related functional changes associated to alterations of the GCM. Moreover, gene expression changes are translated at the protein level, since CB1, CB2 and MOR immunoreactivity in the enteric nervous

system was also modified in the same direction as the gene expression. Furthermore, these changes seem to have functional significance, since visceral pain-related responses were also affected in antibiotic-treated animals. Indeed, visceral pain-related responses elicited by intraperitoneal or direct intracolonic chemical stimulation were attenuated by 40% in antibiotic-treated mice, thus suggesting that treatment with antibiotics can generate an analgesic-like state within the gut. Cannabinoid receptors and MOR are directly involved in visceral pain, eliciting analgesic responses.[39,41] Therefore, the down-regulation of MOR and CB1 contrasts with the analgesic-like state observed, an effect likely compensated by the moderate up-regulation of CB2, which has been implicated in pain modulation in states of inflammation and immune activation.[42] Additionally, NGF has been implicated in the sensitization of visceral afferents leading to the development of hypersensitivity.[43] Therefore, NGF down-regulation might also contribute to the analgesic-like responses observed here.

Recent evidences have linked activation of TLRs, particularly TLR-4, with changes in nociception.[44,45] In the present study, although changes in TLRs expression were relatively minor, we observed correlations between TLR expression (in particular TLR-7) and nociceptive markers (CB2, MOR and TRPV1/3). This furthers supports the possibility that TLRs act as transducers of microbial-generated signals generating local changes in neuro-immune systems and leading to a modulation of viscerosensitivity. Moreover, several studies linking the gut microbiota with visceral sensitivity, have shown that increments in the Lactobacilli family (during probiotic treatment or during states of dysbiosis) are associated to visceral analgesic-like states.[16,17,19,30] In our conditions, we can speculate that the moderate increment in *Lactobacillus* spp. counts observed in antibiotic-treated animals might be important in the observed visceral pain-related responses. Overall these observations might have relevance in IBS patients in which dysbiosis coexists with alterations in visceral sensitivity (visceral hyperalgesia) and this state ameliorates during antibiotic treatment and with administration of certain probiotics.[13,46–49] Although further studies are needed, we can speculate that a similar modulation of sensory-related systems to that described here might operate during antibiotic/probiotic treatments in IBS, leading to an improvement in visceral sensitivity.

The microbiota and microbial-derived products are factors that also affect gastrointestinal motility.[50,51] In the present conditions, basal colonic contractility was increased in dysbiotic mice. This, together with the observed increased responses to carbachol and NO-synthase inhibition

suggests that during dysbiosis enhanced colonic motility represents an imbalance between excitatory (mainly cholinergic) and inhibitory (mainly NO-dependent) systems. Several bacterial metabolic products, such as hydrogen sulphide production or short chain fatty acids, might act as mediators modulating colonic motility. Similarly, it has been suggested that direct TLR4-dependet host-bacterial interactions enhance motility through a neutrally-mediated effect.[50] Overall, these observations suggest that altered gut microbiota might be responsible, at least in part, for the colonic motor disturbances observed in IBS patients and might help to explain the beneficial effects observed after antibiotic or probiotic treatments.[49,52,53]

In summary, the results presented here indicate that during states of dysbiosis there is a local neuro-immune adaptive response, likely associated to changes in host-bacterial interactions, which leads to functional alterations manifested as changes in viscerosensitivity and motor activity within the colon. According to previous observations, we can speculate that proliferation of *C. coccoides*, *Lactobacillus–Enterococcus* spp. and *Bacteroides* spp. and reduction in *Bifidobacterium* spp. counts might be significant for the molecular and functional changes observed. Similar microbial-dependent modulatory actions, might explain the beneficial effects associated to the use of antibiotics or probiotic bacteria in IBS patients.

ACKNOWLEDGMENTS

E. Martínez and A. Acosta are thanked for their technical assistance. Dr. I. Badiola is thanked for their help in the obtention of reference bacterial strains for PCR quantification. Dr. J. Grootjans is thanked for kindly sharing the metha-carnoy protocol.

This study was supported by grants BFU2009-08229 and BES-2010-037699 (FPI program; M. A. personal support) from the Ministerio de Ciencia e Innovación (Spain) and 2009SGR708 from the Generalitat de Catalunya.

The authors don't have any conflict of interest.

REFERENCES

1. Sood R, Law GR, Ford AC. Diagnosis of IBS: symptoms, symptom-based criteria, biomarkers or "psychomarkers"? Nat Rev Gastroenterol Hepatol 2014; (in press; doi: 10.1038/nrgastro.2014.127).

2. Simrén M. IBS with intestinal microbial dysbiosis: a new and clinically relevant subgroup? Gut 2014; (in press; doi: 10.1136/gutjnl-2013-306434).

3. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep 2006;7:688–693.

4. Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? Nat Rev Immunol 2010;10:735–744.

5. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 2008;8:411–420.

6. Collins SM, Bercik P. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology 2009;136:2003–2014.

7. De Palma G, Collins SM, Bercik P, Verdu EF. The microbiota-gut-brain axis in gastrointestinal disorders: stressed bugs, stressed brain or both? J Physiol 2014;592:2989–2997.

8. Koboziev I, Reinoso Webb C, Furr KL, Grisham MB. Role of the enteric microbiota in intestinal homeostasis and inflammation. Free Radic Biol Med 2014;68:122–133.

9. Duerkop BA, Vaishnava S, Hooper L V. Immune responses to the microbiota at the intestinal mucosal surface. Immunity 2009;31:368–376.

10. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol 2013;14:685–690.

11. Foxx-Orenstein AE, Chey WD. Manipulation of the Gut Microbiota as a Novel Treatment Strategy for Gastrointestinal Disorders. Am J Gastroenterol Suppl 2012;1:41–46.

12. Jalanka-Tuovinen J, Salojärvi J, Salonen A, Immonen O, Garsed K, Kelly FM, et al. Faecal microbiota composition and host-microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome. Gut 2013; (in press; doi: 10.1136/gutjnl-2013-305994).

13. Simrén M, Barbara G, Flint HJ, Spiegel BMR, Spiller RC, Vanner S, et al. Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut 2013;62:159–176.

14. Söderholm JD, Yang P-C, Ceponis P, Vohra A, Riddell R, Sherman PM, et al. Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. Gastroenterology 2002;123:1099–1108.

15. Verdú EF, Collins SM. Irritable bowel syndrome and probiotics: from rationale to clinical use. Curr Opin Gastroenterol 2005;21:697–701.

16. Verdú EF, Bercik P, Verma-Gandhu M, Huang X-X, Blennerhassett P, Jackson W, et al. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 2006;55:182–190.

17. Aguilera M, Vergara P, Martinez V. Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. Neurogastroenterol Motil 2013;25:e515–529.

18. Barbara G, Stanghellini V, Brandi G, Cremon C, Di Nardo G, De Giorgio R, et al. Interactions between commensal bacteria and gut sensorimotor function in health and disease. Am J Gastroenterol 2005;100:2560–2568.

19. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 2007;13:35–37.

20. Collins S, Verdé E, Denou E, Bercik P. The role of pathogenic microbes and commensal bacteria in irritable bowel syndrome. Dig Dis 2009;27 Suppl 1:85–89.

21. Goto Y, Ivanov II. Intestinal epithelial cells as mediators of the commensal-host immune crosstalk. Immunol Cell Biol 2013;91:204–214.

22. Salzman NH, Bevins CL. Dysbiosis—A consequence of Paneth cell dysfunction. Semin Immunol 2013;25:334–341.

23. Saulnier DM, Ringel Y, Heyman MB, Foster JA, Bercik P, Shulman RJ, et al. The intestinal microbiome, probiotics and prebiotics in neurogastroenterology. Gut Microbes 2013;4:17–27.

24. Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, et al. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. Infect Immun 2011;79:1536–1545.

25. Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, Kirn TJ, et al. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol 2010;3:148–158.

26. Puhl NJ, Uwiera RRE, Yanke LJ, Selinger LB, Inglis GD. Antibiotics conspicuously affect community profiles and richness, but not the density of bacterial cells associated with mucosa in the large and small intestines of mice. Anaerobe 2012;18:67–75.

27. Croswell A, Amir E, Teggatz P, Barman M, Salzman NH. Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. Infect Immun 2009;77:2741–2753.

28. Dinan TG, Cryan JF. Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology 2012;37:1369–1378.

29. Barbara G, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? Gut 2002;51 Suppl 1:i41–44.

30. Xu D, Gao J, Gillilland M, Wu X, Song I, Kao JY, et al. Rifaximin alters intestinal bacteria and prevents stress-induced gut inflammation and visceral hyperalgesia in rats. Gastroenterology 2014;146:484–496.

31. Barnes MJ, Powrie F. Immunology. The gut's Clostridium cocktail. Science 2011;331:289–290.

32. Koboziev I, Reinoso Webb C, Furr KL, Grisham MB. Role of the enteric microbiota in intestinal homeostasis and inflammation. Free Radic Biol Med 2013;68C:122–133.

33. Swidsinski A, Loening-Baucke V, Lochs H, Hale L-P. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World J Gastroenterol 2005;11:1131–1140.

34. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 2010;10:131–144.

35. Terán-Ventura E, Aguilera M, Vergara P, Martínez V. Specific changes of gut commensal microbiota and TLRs during indomethacin-induced acute intestinal inflammation in rats. J Crohns Colitis 2014;8:1043-1054.

36. Terán-Ventura E, Roca M, Martin MT, Abarca ML, Martinez V, Vergara P. Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats. Microb Ecol 2010;60:691–702.

37. Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, et al. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PLoS One 2011;6:e17996.

38. Deplancke B, Gaskins HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am J Clin Nutr 2001;73:1131S–1141S.

39. Boué J, Basso L, Cenac N, Blanpied C, Rolli-Derkinderen M, Neunlist M, et al. Endogenous regulation of visceral pain via production of opioids by colitogenic CD4(+) T cells in mice. Gastroenterology 2014;146:166–175.

40. Distrutti E, Cipriani S, Mencarelli A, Renga B, Fiorucci S. Probiotics VSL#3 protect against development of visceral pain in murine model of irritable bowel syndrome. PLoS One 2013;8:e63893.

41. Brusberg M, Arvidsson S, Kang D, Larsson H, Lindström E, Martinez V. CB1 receptors mediate the analgesic effects of cannabinoids on colorectal distension-induced visceral pain in rodents. J Neurosci 2009;29:1554–1564.

42. Wright KL, Duncan M, Sharkey KA. Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation. Br J Pharmacol 2008;153:263–270.

43. Jardí F, Fernández-Blanco JA, Martinez V, Vergara P. Plasticity of Dorsal Root Ganglion Neurons in a Rat Model of Post-Infectious Gut Dysfunction: Potential Implication of Nerve Growth Factor. Scand J Gastroenterol 2014; (in press).

44. Meseguer V, Alpizar YA, Luis E, Tajada S, Denlinger B, Fajardo O, et al. TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. Nat Commun 2014;5:3125.

45. Tramullas M, Finger BC, Moloney RD, Golubeva A V, Moloney G, Dinan TG, et al. Toll-like receptor 4 regulates chronic stress-induced visceral pain in mice. Biol Psychiatry 2014;76:340–348.

46. Cash BD. Emerging role of probiotics and antimicrobials in the management of irritable bowel syndrome. Curr Med Res Opin 2014;30:1405–1415.

47. Guglielmetti S, Mora D, Gschwender M, Popp K. Randomised clinical trial: Bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life--a double-blind, placebo-controlled study. Aliment Pharmacol Ther 2011;33:1123–1132.

48. Koo HL, DuPont HL. Rifaximin: a unique gastrointestinal-selective antibiotic for enteric diseases. Curr Opin Gastroenterol 2010;26:17–25.

49. Pimentel M, Chow EJ, Lin HC. Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. Am J Gastroenterol 2000;95:3503–3506.

50. Anitha M, Vijay-Kumar M, Sitaraman S V, Gewirtz AT, Srinivasan S. Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. Gastroenterology 2012;143:1006–1016.

51. Bär F, Von Koschitzky H, Roblick U, Bruch HP, Schulze L, Sonnenborn U, et al. Cell-free supernatants of Escherichia coli Nissle 1917 modulate human colonic motility: evidence from an in vitro organ bath study. Neurogastroenterol Motil 2009;21:559–66.

52. Guyonnet D, Chassany O, Ducrotte P, Picard C, Mouret M, Mercier C-H, et al. Effect of a fermented milk containing Bifidobacterium animalis DN-173 010 on the health-related quality

of life and symptoms in irritable bowel syndrome in adults in primary care: a multicentre, randomized, double-blind, controlled trial. Aliment Pharmacol Ther 2007;26:475–486.

53. Quigley EMM. Bacteria: a new player in gastrointestinal motility disorders--infections, bacterial overgrowth, and probiotics. Gastroenterol Clin North Am 2007;36:735–748.

54. Van der Waaij D, Berghuis-de Vries JM, Korthals Altes C. Oral dose and faecal concentration of antibiotics during antibiotic decontamination in mice and in a patient. J Hyg (Lond) 1974;73:197–203.

55. Aguilera M, Vergara P, Martinez V. Environment-related adaptive changes of gut commensal microbiota do not alter colonic toll-like receptors but modulate the local expression of sensory-related systems in rats. Microb Ecol 2013;66:232–243.

56. Furet J-P, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, et al. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol Ecol 2009;68:351–362.

57. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, et al. Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PLoS One 2011;6:e28654.

58. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408.

59. Martinez V, Thakur S, Mogil JS, Taché Y, Mayer E a. Differential effects of chemical and mechanical colonic irritation on behavioral pain response to intraperitoneal acetic acid in mice. Pain 1999;81:179–186.

60. Harmsen HJM, Raangs GC, He T, Degener JE, Welling GW. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl Environ Microbiol 2002;68:2982–2990.

61. Haarman M, Knol J. Quantitative real-time PCR analysis of fecal Lactobacillus species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 2006;72:2359–2365.

62. Salzman NH, de Jong H, Paterson Y, Harmsen HJM, Welling GW, Bos NA. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiology 2002;148:3651–3660.

63. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjöberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 2010;11:76–83.

GENERAL DISCUSSION

During the last years, the microbial compartment has been described as a key factor contributing to both intestinal health and disease states. Imbalances between the microbial, the immune and the nervous systems, which are supposed to maintain the GI milieu, seem to be causative factors of gut diseases. In particular, alterations in the normal composition of the GCM (dysbiosis) have been suggested to be a pathogenic factor in functional GI disorders, specifically in IBS. IBS is considered a multifactorial disorder comprising genetic susceptibilities, chronic stress, enteric infections, deregulations of the brain-gut axis, altered intestinal immune function, motility and visceral sensitivity and an altered microbial composition (Longstreth *et al.*, 2006; Khan and Chang, 2010; Simrén *et al.*, 2013). The cause-effect relationship among these factors remains unknown and it is not clear if the dysbiotic state described in IBS patients is a cause or a consequence or how the dysbiotic state contributes to the pathophysiology of the disease.

In order to gain insight into the potential role of microbiota in IBS, and particularly its implication in visceral pain-related sensory alterations we assessed the expression of colonic sensory systems and changes in visceral pain in different situations of colonic dysbiosis in rodents. Moreover, to further explore the mechanisms involved, we also studied dysbiosis-associated changes in the local innate immune system and in host-bacterial interaction systems.

Composition of the gut commensal microbiota is similar in rats and mice and follows the same architecture described in humans

Although it was not a primary objective of this work, the studies performed confirm previous data showing that Gram positive Firmicutes, specifically the groups belonging to the *Clostridium* clusters XIVa and XIVb, followed by the Gram negative *Bacteroides* spp., represent the vast majority of the GCM, either in rats or mice. These groups represented, overall, by 90% of the total bacterial counts, in agreement with previous reports (Dinoto *et al.*, 2006; Terán-Ventura *et al.*, 2010; Hildebrand *et al.*, 2013). Other groups assessed (Verrucobacteria, Enterobacteria, Lactobacilli and Bifidobacteria) were scarce or even in the limits of detection. Despite the presence of relative and specific fluctuations in the microbial composition due to the different treatments (e.g. antibiotics) or environmental conditions, the described common architecture of the commensals, in terms of bacterial families, were stable in all animals. Interestingly, from a relative

point of view, the groups less represented (Enterobacteria, Lactobacilli and Bifidobacteria) were, overall, the more susceptible to change in the course of dysbiosis.

Taking into account the recently introduced concept of enterotypes, this general structure of the GCM follows the same pattern as that described in humans (Eckburg *et al.*, 2005; Arumugam *et al.*, 2011). This reinforces the translational validity of animal studies as predictors of human responses related to the gut microbiota.

Spontaneous housing-related changes in commensal microbiota are not enough to change the expression of colonic TLR-dependent host-bacterial interaction systems but influence the expression of colonic nociceptive markers

We have previously described that the microbial compartment, although its stability, is a dynamic system that can be influenced by the housing environmental conditions (Terán-Ventura *et al.*, 2010). Following that approach, we characterized how spontaneous environmental-related adaptive changes might affect sensory-relayed systems within the gut. In agreement with previous observations, the adaptation from a barrier environment (high degree of hygiene with reduced bacterial charge) to a conventional environment (lower degree of hygiene with increased bacterial charge) presented a change in the microbiota towards the characteristics of those animals born and breed in conventional conditions. Main adaptive changes included specific variations in *Bifidobacterium* spp., *Bacteroides* spp. and *Lactobacillus-Enterococcus* spp. groups, without changes in total bacterial counts. In these animals, TLRs expression was not affected, thus suggesting that the microbial changes were not enough to alter host-bacterial interaction systems, at least as it relates to TLRs expression. However, in the same animals the expression of different sensory-related markers, namely TRPV3, MOR and CB2, was down-regulated, approaching the situation reflected in animals breed in conventional conditions. Although the changes observed were limited, this represents a clear indication that the microbiota can affect sensory mechanisms within the gut. Nevertheless, we wanted to explore dysbiotic states in which the microbial changes were associated to an immune activation (at least as it relates to host-bacterial interaction systems) to be more closely related to the situations described in IBS.

Simulation of a dysbiotic state with altered TLR4- and 7-dependent signaling produces a local immune activation without changes in the microbial composition

With the intention of generating a microbial-related local immune response we mimetized a state of dysbiosis with altered (increased) host-bacterial interactions by means of a direct stimulation of colonic TLRs. Specifically, we simulated a dysbiosis by over-stimulation of TLR4 and 7, using selective ligands for these receptors, bacterial lipopolysaccharide (LPS) and imiquimod (IMQ), respectively (Poltorak *et al.*, 1998; Lee *et al.*, 2003; Abreu, 2010).

As expected, direct stimulation of TLRs elicited a local response in the host that was TLR- and duration of treatment-related. Overall, responses observed were minor after a single treatment but increased in magnitude and in the number of parameters affected during a 5-day repeated treatment. This is consistent with the idea that a sustained (chronic) dysbiosis will elicit stronger responses that just a short, transitory, microbial change. In fact, in the same experimental conditions, no changes were observed when the same parameters were evaluated 5-h after TLRs stimulation (data not included). Overall, an immune activation characterized by an up-regulation of pro- and anti-inflammatory cytokines was observed. This agrees with the responses described using a similar approach in both *in vivo* and *in vitro* situations (Gaekwad *et al.*, 2010; Sainathan *et al.*, 2012; Yue *et al.*, 2012; Gutschow *et al.*, 2013). Moreover, systems directly implicated in host-bacterial interactions were also up-regulated. These included the expression of integrins, antimicrobial peptides and the self-regulation of TLRs as well as changes in s-IgA and the ratio of s-IgA-coated bacteria. All together, these changes are consistent with a state in which host-bacterial interactions might be increased.

As mentioned, changes observed were also TLR-dependent, with simulation of TLR4 with LPS leading to more extended effects than those observed after TLR7 stimulation with imiquimod. Since TLR4 are stimulated by lipopolysaccharides produced by Gram-negative bacteria (Munford, 2008), this might suggest that the presence in the intestine of LPS-producing Gram-negative bacteria, such as some pathogenic strains of *E. coli*, might be particularly important eliciting immune responses in the host. Dysbiotic states involving these bacterial groups might have relevant pathophysiological implications.

Taking into account that host-bacterial interactions seem to be bidirectional we also expected to find changes in the microbial composition during TLRs stimulation. However, both

luminal and wall adhered microbiota were fairly constant, regardless the treatment considered. Nevertheless, from the present observations we cannot exclude that in conditions in which different TLRs and/or other PRRs are stimulated the responses elicited by the host might be able to affect the microbiota. Indeed, it has been suggested that part of the host-bacterial interactions associated to a dysbiotic state, particularly those generated by the host, are directed towards the reshaping of the microbiota and the restoration of the eubiotic state (Fung *et al.*, 2014; Kamada and Núñez, 2014; Peterson and Artis, 2014).

Antibiotic-induced dysbiosis produces a local immune activation

Finally, taking into account that other approaches failed inducing a dysbiotic state, animals were treated with non-absorbable, broad-spectrum antibiotics. In these conditions, we were able to observe a real dysbiotic state, with changes in the total bacterial load and the relative composition of the colonic microbiota, with changes in the counts of specific bacterial groups. The dysbiosis generated, as it relates to both luminal and wall adhered microbiota, was related to the duration of the treatment (1-week vs. 2-week) and was consistent with previous studies in rats and mice (Verdú *et al.*, 2006; Croswell *et al.*, 2009; Hill *et al.*, 2010; Wlodarska *et al.*, 2011). Moreover, changes in bacterial morphology, particularly for the *C. coccoides* group (cluster XIVa), were also observed, thus suggesting that antibiotics might promote the expansion of some bacterial groups, likely resistant to the antibiotic treatment.

In these conditions, significant changes in innate immune components and host-bacterial interaction systems were observed. Selective up- and down-regulations were observed depending upon the component considered. Changes observed affected also the mucus layer, likely facilitating bacterial adherence and, thus, enhancing bacterial-derived signaling to the host and subsequent host responses. As discussed above, changes observed suggest the existence of an overall host response directed towards the reshaping of the microbiota and the restoration of an eubiotic state.

Immune activation due to microbial changes does not cause an overt colonic inflammation

Traditionally, opportunistic pathogens and alterations of the commensal bacteria have been linked to intestinal inflammation (Sartor and Mazmanian, 2012). Moreover, dysbiosis is a common observation in IBD and IBS patients and a feature of animals models of these diseases (Rodríguez-Fandiño *et al.*, 2010; Spiller and Lam, 2011; Chassard *et al.*, 2012; O'Mahony *et al.*, 2014; Schwab *et al.*, 2014; Terán-Ventura *et al.*, 2014). However, as mentioned, a cause-effect relationship has not been clearly established. In the present studies, an activation of the local immune system (more or less general depending upon de model considered) was observed. However, regardless the model considered (species, mouse vs. rats; or the treatments applied) or the extent of the local immune activation observed, in no case, macroscopical or microscopical signs of colonic inflammation were observed. Such a state might be consistent with that described in IBS patients in which an immune activation is described in the absence of structural alterations consistent with the existence of inflammation, defined by some authors as an state of low inflammatory degree (Bercik *et al.*, 2005; Akiho *et al.*, 2010; Collins, 2014; Piche, 2014). These similarities will reinforce the translational value of the observations derived from animal models of dysbiosis to the human condition.

Microbiota influences intestinal sensory-related systems implicated in viscerosensitivity

Changes in visceral sensitivity are a common finding in IBS patients; in fact, visceral hypersensitivity is considered one of the main symptoms of IBS (Sikandar and Dickenson, 2012; Fukudo, 2013). During the last years some reports suggest that microbiota might influence sensory mechanisms within the gut leading to altered visceral pain-related responses (Parkes *et al.*, 2008; Collins *et al.*, 2009; Lee and Lee, 2014; Theodorou *et al.*, 2014). Since IBS patients, together with other alterations, present also a dysbiotic situation (Rajilić-Stojanović *et al.*, 2011; Jalanka-Tuovinen *et al.*, 2013; Simrén *et al.*, 2013), it seems feasible that a link between the microbiota and the manifestations of the disease (such as changes in viscerosensitivity) should exist. To gain insight in this aspect, a main objective of this work was to assess changes in colonic sensory-related systems in the different dysbiotic states generated.

In all cases, the perturbation of the microbiota (either spontaneous alterations during an adaptive process or alterations induced with antibiotics) or the stimulation of host-bacterial interactions systems (in the absence of a manifest dysbiotic state) were associated to changes in the local expression of sensory-related markers. Interestingly, both antinociceptive systems (such as the endocannabinoid and the opioid systems) and pro-nociceptive systems (such as TRPV) were affected. However, the pattern of change was not constant and selective up- or down-regulations (with higher or lower immunorreactivity at the level of the ENS) were observed depending upon the experimental situation considered. From these observations, it is difficult to speculate on the final functional consequences, if any, of these changes. Simultaneous up- and down-regulation of pro- and anti-nociceptive systems might be a consequence of the redundancy in regulatory mechanisms and might suggest a fine tuning in pain-related responses, with functional consequences depending upon the balance between proalgesic and analgesic mechanisms. In any case, these observations strongly support the existence of a modulation of sensory systems during states of dysbiosis (real or perceived by the host). Moreover, changes in NGF expression, observed in some of the conditions studied, further support the existence of a neuronal remodeling, likely affecting sensory pathways. Neurotrophins, and particularly NGF, have been implicated in the remodeling of neural pathways in states of inflammation and immune activation; and have been associated with changes in nociceptive responses (Barreau *et al.*, 2004; Stanzel *et al.*, 2008; Hoffman *et al.*, 2011; Tsang *et al.*, 2012; Jardí *et al.*, 2014; Lewin *et al.*, 2014).

From the present studies, we cannot define the mechanisms underlying these changes in sensory markers. Nevertheless, we can speculate that direct interaction of microbial components with the host (for instance via TLRs) might be important. Observations in other systems have shown that stimulation of TLRs initiates complex signaling cascades that lead to the local release of neuro-immune mediators (Kraneveld *et al.*, 2008; Rodríguez-Fandiño *et al.*, 2010; Hughes *et al.*, 2013; Assas *et al.*, 2014). In these conditions, the remodeling of sensory mechanisms is likely to occur, as described for states of inflammation (Bueno and Fioramonti, 2002; Fernández-Blanco *et al.*, 2011; Demir *et al.*, 2013). Furthermore, TLRs, an in particular TLR4, have been directly associated with the modulation of visceral pain arising from the gut (Meseguer *et al.*, 2014; Sauer *et al.*, 2014; Tramullas *et al.*, 2014), further establishing a direct link between microbiota and sensory mechanisms.

Some *in vitro* and *in vivo* studies have linked specific bacterial strains with potential actions (beneficial or harmful) within the gut. In our studies, it is difficult to establish a direct link between a particular bacterial group and the effects observed in sensory systems. Although some of the changes observed in sensory markers correlated with variations in the counts of some specific bacterial groups, a cause-effect relationship cannot be established. Since our approach was based on relatively large variations of the GCM as a whole, the changes observed are likely to result from the interaction of multiple bacterial groups. Nevertheless, bacterial groups present in low percentages, such as the Lactobacilli-Enterococci group, seem to be the ones more implicated with changes in the expression of nociceptive markers. However, the largest commensal group, the Clostridia cluster XIVa group, also showed correlations with nociceptive markers. Future studies assessing particular bacterial groups/strains, used as a probiotic treatment, and/or axenic animals might clarify these aspects and delimitate the exact components of the GCM with the ability to affect sensory mechanism within the gut.

Microbial-induced changes in sensory systems translate into attenuated visceral pain-related responses and altered colonic contractility

Mice with antibiotic-induced dysbiosis showed attenuated visceral pain-related responses in two models of chemically-induced visceral pain, the Writhing test and the intracolonic administration of capsaicin. These observations give a functional significance to the molecular changes in sensory-related systems discussed above. Overall, these data agree with previous reports showing that visceral pain responses were modified in states of dysbiosis or by the treatment with certain probiotic strains (Verdú *et al.*, 2006; Rousseaux *et al.*, 2007; Amaral *et al.*, 2008; O'Mahony *et al.*, 2014).

Similar attenuation in visceral pain-related responses were observed after a 1-week or 2-week antibiotic treatment. However, the dysbiotic state generated and the changes in sensory-related markers were not identical in both situations (1-week vs. 2-week treatment). This might indicates that the functional effects observed do not result neither from the modulation of a particular sensory mechanisms nor from changes in a particular bacterial group. Indeed, the antinociceptive responses are likely to reflect the balance of simultaneous changes in several sensory systems (mainly endocannabinoid, opioid and vanilloid) associated to multiple combined

changes of the GCM. As mentioned above, both analgesic (endocannabinoids and opioids) and proalgesic (vanilloid) systems were modulated during antibiotics-induced dysbiosis, further indicating that the final analgesic-like responses observed should results from a balance between mechanism promoting and containing pain.

Antibiotic-induced dysbiosis in mice was also associated to changes in colonic contractility, as assessed *in vitro* in an organ bath system. In these conditions, spontaneous smooth muscle activity and responses to the cholinergic stimulation were increased in dysbiotic animals, thus suggesting an increased excitatory state that, in *in vivo* conditions, should results in increased colonic motility. Indeed, previous studies implicated cholinergic-dependent mechanisms in the motility effects associated to bacterial-derived products (Guyonnet *et al.*, 2007; Anitha *et al.*, 2012; Reigstad and Kashyap, 2013). However, the NO-dependent inhibitory component was also increased in dysbiotic animals. This might represent a compensatory mechanism to the state of hyperactivity described above. In fact, colonic motility seem to be normal in animals with antibiotic-induced dysbiosis, at least as we have observed taking fecal pellet output as a measure of colonic motor activity. However, differences in the experimental protocols (mainly the duration of the treatment with antibiotics) should be taking into account.

In a state of antibiotics-induced dysbiosis endocrine and colonic motor responses to stress are not altered

Recent evidences support the existence of a brain-gut-microbiota axis. This is evidenced by studies indicating that the gut microbiota can influence CNS functions and might participate in neural pathologies (Rhee *et al.*, 2009; Dinan and Cryan, 2012; Foster and McVey Neufeld, 2013; Montiel-Castro *et al.*, 2013). Among other effect, the microbiota could be able to modulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis and, through this, modulate the responses to stress (Grenham *et al.*, 2011). As discussed along this work, both stress and microbiota are significant pathogenic components of IBS (Taché et al., 2004; Lutgendorff et al., 2008; Gulewitsch et al., 2013; Jalanka-Tuovinen et al., 2013; Kennedy et al., 2014), therefore their interaction might be important determining the characteristics and course of the disease.

To gain insight into the interplay stress-microbiota we combined an state of antibiotic-induced dysbiosis with psychological stress (repeated water avoidance stress) in mice.

Psychological stress, *per se*, induced a characteristic dysbiotic state, different to that associated to antibiotics, thus suggesting and activation of the brain-to-gut signaling results in a microbial modulation. Stress also modulated colonic sensory-related systems, an effect that was further enhanced by the treatment with antibiotics, resulting, in particular, in an up-regulation of CB2 receptors. These interactions were also evidenced at a functional level, leading to a modulation of visceral pain-related responses. As expected from previous works, stress increased visceral sensitivity (Bradesi *et al.*, 2002; Posserud *et al.*, 2004) and the addition of antibiotics prevented these effect. Overall, this might suggest an interaction between luminal microbial-derived and stress-derived CNS signals modifying sensory mechanism within the gut. However, despite these interactions, the endocrine (as it relates to activation of the HPA axis) and colonic motor responses to stress were not affected by antibiotics.

Overall, these observations support the existence of a brain-gut-microbiota axis and reinforce the view that stress and the gut microbiota should be regarded as interacting factors in the pathophysiology of functional GI disorders.

Figure 6 integrates the findings of the present studies with literature data and shows the main components of the brain-gut-microbiota-axis, highlighting some of the mechanisms through which intestinal microbiota could affect visceral pain-related responses. Microbiota and microbial-derived products are likely to interact with host bacterial recognition systems (such as TLRs) leading to a signaling cascade that will lead to a local immune activation. This implies the generation of a host response involving a series of mechanisms (secretion of IgA, modulation of AMPs and integrins expression, changes in the mucus layer, etc.) likely directed towards the reshaping of the microbiota. In these conditions, TLR and immune signals might influence sensory neural mechanisms within the gut, et the level of the ENS and the extrinsic innervation. Sensory systems affected seem to involve, at least but not exclusively, the endocannabinoid, opioid, and vanilloid systems. In this conditions, bidirectional communication with the CNS, will lead to an altered perception of sensory signals and an altered manifestation of pain. Changes in pain responses observed here suggest, in fact, that during antibiotic-induced dysbiosis this circuit is remodeled as to elicit analgesic responses against, at least, the chemical noxious stimulation of the colon. This might represent a protective mechanism of the body to avoid enhanced pain responses in states of alteration of the gastrointestinal homeostasis.

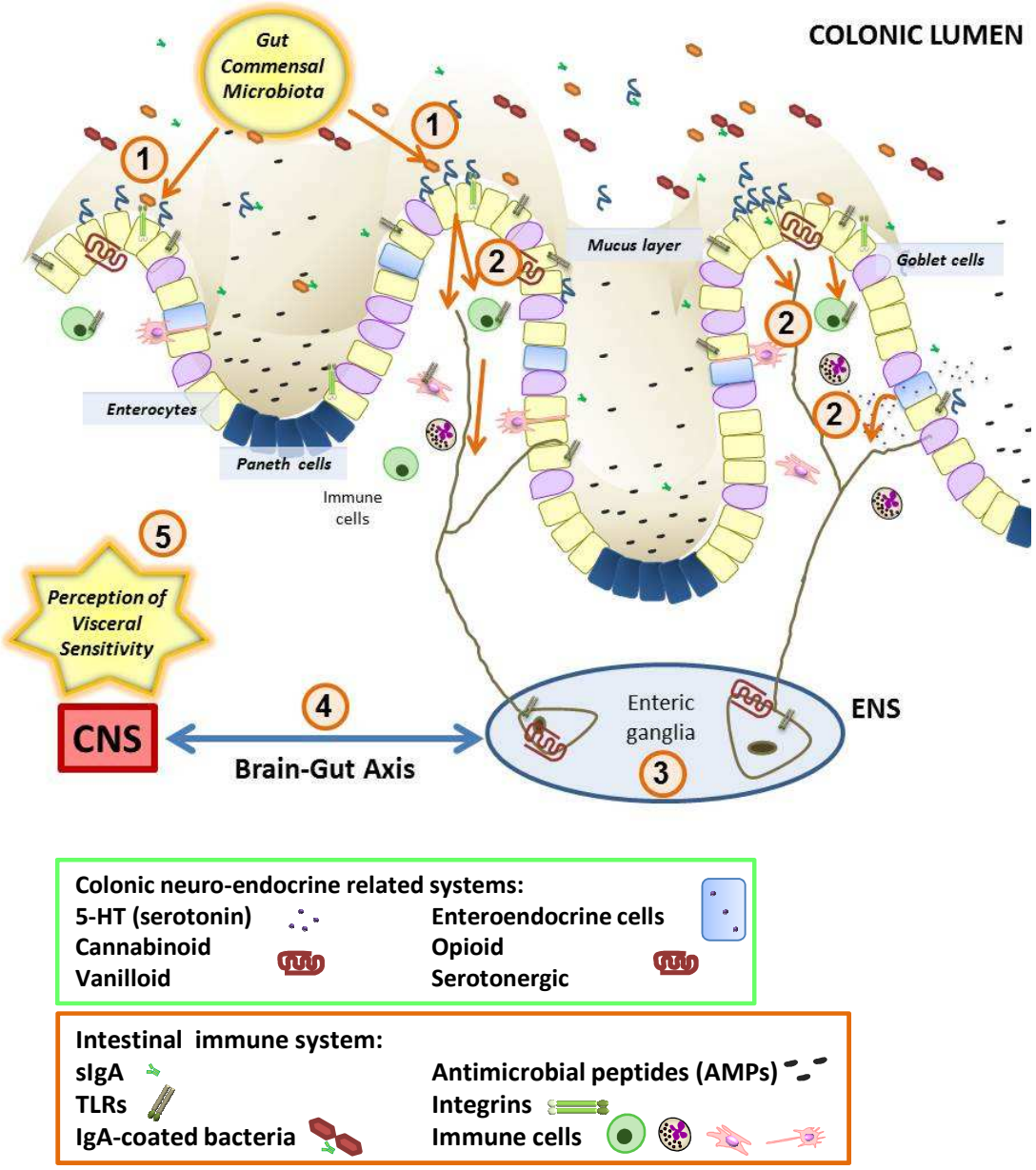


Figure 6. Schematic representation of some of the putative mechanisms leading to microbial-mediated changes in visceral sensitivity. Microbiota and microbial-derived products interact with host bacterial recognition systems (such as TLRs) (1) generating to a signaling cascade (2) that will lead to a local immune activation. TLRs and immune signals (2) might influence sensory neural mechanisms within the gut, depending upon the ENS (3) and the extrinsic innervation. In this conditions, bidirectional communication with the CNS (4), will lead to an altered perception of sensory signals and an altered manifestation of pain (5).

In summary, the results obtained in this work give a general overview of the impact of GCM on intestinal neuro-immune interactions, with emphasis in sensory-related mechanisms, from a molecular to a functional point of view. We have shown that the GCM microbiota is a dynamic organ directly implicated in host pathophysiological neuro-immune responses. Dysbiotic states (including altered signaling from microbial recognition systems) induce a local immune activation, compatible with the recently described state of low-grade inflammation in IBS patients, modulate the expression of sensory-related markers involved in visceral pain perception and lead to altered functional responses in visceral pain. Given the fact that the microbiota is altered in functional GI disorders, and particularly in IBS patients, and that altered visceral sensitivity (hypersensitivity) is a major finding in the same subjects, the present data further support the view that the GCM represents a highly attractive intervention target for the treatment of IBS.

CONCLUSIONS

1. In rats, environment-related spontaneous adaptive changes of the colonic commensal microbiota do not affect the expression of toll-like receptor-dependent host-bacterial interaction systems, at list as it relates to Toll-like receptors 2, 4, 5 and 7; but down-regulate colonic sensory-related systems; particularly mu-opioid receptors; and to a lesser extend the cannabinoid receptor type 2 and the protease-activate receptor 2.

2. In rats, the simulation of a colonic dysbiotic state by direct stimulation of colonic Toll-like receptors 4 and 7 induces a time-related and toll-like receptor-specific host response directed towards the modulation of the microbiota. This response includes a local immune activation characterized by an increase in the counts of IgA-coated bacteria and an up-regulation of antimicrobial peptides, integrins, pro- and anti-inflammatory cytokines and toll-like receptors.

3. In rats, the stimulation of colonic Toll-like receptor 4, but not Toll-like receptor 7, leads to a local modulation of sensory-related systems characterized by an up-regulation of cannabinoid receptors type 1 and 2 and transient receptor potential vanilloid types 1, 3 and 4.

4. In mice, repeated psychological stress generates a colonic dysbiotic state characterized by an increment in the luminal counts of *Clostridium* cluster XIVa and a decrease in luminal and wall-adhered Verrucobacteria.

5. In mice, endocrine and colonic motor responses to repeated psychological stress are not altered during states of antibiotic-induced dysbiosis; revealing a normal functionality of the gut-brain-gut axis.

6. In mice, antibiotic-induced colonic dysbiosis depends upon the duration of the treatment. A 1-week antibiotic exposure reduces total bacterial load, with a combined increase in *Lactobacillus-Enterococcus* spp. and Enterobacteria and a reduction in *Clostridium* cluster XIVa counts. A 2-week antibiotic exposure increases total bacterial load, with a combined reduction in *Bacteroides* spp. and an increase in *Bifidobacterium* spp. and *Clostridium coccoides* counts.

7. In mice, bacterial adherence to the colonic epithelium is favored during a 1-week, but not a 2-week, antibiotic treatment. The addition of psychological stress further enhances *Bacteroides* spp. attachment while reducing the adherence of Enterobacteria.

Conclusions

8. In mice, antibiotic-induced colonic dysbiosis modulates the local immune system; with an increase in luminal levels of s-IgA and a selective up- or down-regulation of antimicrobial peptides and toll-like receptors.

9. In mice, antibiotic-induced colonic dysbiosis leads to the local modulation of sensory related systems. Specifically, an up-regulation of the cannabinoid receptor type 2 and a down-regulation of the cannabinoid receptor type 1 and mu-opioid receptors are observed. These changes translate into attenuated visceral pain-related responses and enhanced colonic contractility.

10. Neither antibiotic-induced dysbiosis in mice nor the simulation of a dysbiotic state using selective ligands for toll-like receptor 4 or 7 in rats cause macroscopical or microscopical signs of colonic inflammation.

CONCLUSIONES

1. En la rata, los cambios espontáneos de la microbiota comensal del colon asociados a la adaptación a nuevas condiciones ambientales no afectan la expresión de los sistemas de interacción hospedador-microbiota dependientes de receptores de tipo Toll, al menos en lo que se refiere a los subtipos 2, 4, 5 y 7; pero implican una regulación a la baja de la expresión cólica de sistemas relacionados con funciones sensoriales; principalmente de los receptores opioides de tipo mu, y en menor grado de los receptores canabinoides de tipo 2 y del receptor activado por proteasas de tipo 2.

2. En la rata, la simulación de un estado de disbiosis cólica mediante la estimulación directa de los receptores de tipo Toll 4 y 7 induce una respuesta dirigida al control de la microbiota que depende tanto del receptor estimulado como de la duración de dicha estimulación. Esta respuesta incluye una activación local del sistema inmune caracterizada por un aumento en los contajes de bacterias cubiertas de IgA y una regulación al alza de la expresión de péptidos antimicrobianos, integrinas, citoquinas pro- y anti-inflamatorias y de los propios receptores de tipo Toll.

3. En la rata, la estimulación del receptor de tipo Toll 4 del colon, pero no del 7, produce una modulación de los sistemas sensoriales locales; caracterizada por una regulación al alza de los receptores canabinoides de tipo 1 y 2 y de los receptores de potencial transitorio V1, V3 y V4.

4. En el ratón, el estrés psicológico repetido genera un estado de disbiosis cólica caracterizado por un incremento en los recuentos luminales de *Clostridium* grupo XIVa y una disminución en los recuentos luminales y la adherencia epitelial del grupo Verrucobacteria.

5. En el ratón, la respuestas endocrinas y motoras del colon asociadas al estrés psicológico repetido no se ven alteradas durante estados de disbiosis inducidos con antibióticos; mostrando una funcionalidad normal del eje intestino-cerebro-intestino.

6. En el ratón, la disbiosis cólica inducida por el tratamiento con antibióticos depende de la duración del mismo. La administración de antibióticos durante 1 semana reduce la carga bacteriana total, con una reducción en los contajes de *Clostridium* cláster XIVa y un aumento simultáneo en los recuentos de *Lactobacillus-Enterococcus* spp. y Enterobacteria. La administración de antibióticos durante 2 semanas aumenta la carga bacteriana total, con un aumento en los recuentos de *Bifidobacterium* spp. y *Clostridium coccoides* y una reducción simultánea en los recuentos de *Bacteroides* spp.

Conclusiones

7. En el ratón, la adherencia bacteriana al epitelio del colon aumenta tras el tratamiento con antibióticos durante 1 semana, pero no durante 2 semanas. La adición de estrés psicológico al tratamiento con antibióticos facilita la adherencia de *Bacteroides* spp. y reduce la adherencia del grupo Enterobacteria.

8. En el ratón, la disbiosis cólica inducida con antibióticos modula el sistema inmune local; observándose un aumento en la secreción de IgA y una regulación selectiva al alza o a la baja de péptidos antimicrobianos y de receptores de tipo Toll.

9. En el ratón, la disbiosis cólica inducida con antibióticos resulta en una modulación de la expresión de los sistemas sensoriales locales; regulando al alza la expresión del receptor canabinoide de tipo 2 y a la baja la expresión del receptor canabinoide de tipo 1 y de los receptores opioides de tipo mu. Estos cambios moleculares se traducen a nivel funcional en respuestas de dolor visceral atenuadas y en un aumento de la contractilidad cólica.

10. Ni la disbiosis cólica inducida con antibióticos en el ratón, ni su simulación por estimulación local de los receptores de tipo Toll 4 o 7 con ligandos selectivos en la rata causa signos macroscópicos o microscópicos de inflamación en el colon.

REFERENCES

Abreu MT (2010) Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 10:131–144.

Akiho H, Ihara E, Nakamura K (2010) Low-grade inflammation plays a pivotal role in gastrointestinal dysfunction in irritable bowel syndrome. World J Gastrointest Pathophysiol 1:97–105.

Albiger B, Dahlberg S, Henriques-Normark B, Normark S (2007) Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. J Intern Med 261:511–528.

Alhouayek M, Muccioli GG (2012) The endocannabinoid system in inflammatory bowel diseases: from pathophysiology to therapeutic opportunity. Trends Mol Med 18:615–625.

Amaral FA, Sachs D, Costa V V, Fagundes CT, Cisalpino D, Cunha TM, Ferreira SH, Cunha FQ, Silva TA, Nicoli JR, Vieira LQ, Souza DG, Teixeira MM (2008) Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 105:2193–2197.

Anitha M, Vijay-Kumar M, Sitaraman S V, Gewirtz AT, Srinivasan S (2012) Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. Gastroenterology 143:1006–1016.

Arumugam M et al. (2011) Enterotypes of the human gut microbiome. Nature 473:174–180.

Assas BM, Miyan JA, Pennock JL (2014) Cross-talk between neural and immune receptors provides a potential mechanism of homeostatic regulation in the gut mucosa. Mucosal Immunol. doi: 10.1038/mi.2014.80.

Atreya R, Neurath MF (2008) New therapeutic strategies for treatment of inflammatory bowel disease. Mucosal Immunol 1:175–182.

Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 25:397–407.

Bailey MT, Engler H, Sheridan JF (2006) Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. J Neuroimmunol 171:29–37.

Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, Rumio C (2009) Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. J Histochem Cytochem 57:1013–1023.

Barker N (2014) Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. Nat Rev Mol Cell Biol 15:19–33.

Barnes MJ, Powrie F (2011) Immunology. The gut's Clostridium cocktail. Science 331:289–290.

Barnett AM, Roy NC, McNabb WC, Cookson AL (2012) The interactions between endogenous bacteria, dietary components and the mucus layer of the large bowel. Food Funct 3:690–699.

Barreau F, Cartier C, Ferrier L, Fioramonti J, Bueno L (2004) Nerve growth factor mediates alterations of colonic sensitivity and mucosal barrier induced by neonatal stress in rats. Gastroenterology 127:524–534.

Baumgart DC, Sandborn WJ (2012) Crohn's disease. Lancet 380:1590–1605.

Beaulieu JF (1999) Integrins and human intestinal cell functions. Front Biosci 4:D310–321.

Bemark M, Boysen P, Lycke NY (2012) Induction of gut IgA production through T cell-dependent and T cell-independent pathways. Ann N Y Acad Sci 1247:97–116.

Bercik P, Verdu EF, Collins SM (2005) Is irritable bowel syndrome a low-grade inflammatory bowel disease? Gastroenterol Clin North Am 34:235–245.

Billiet T, Rutgeerts P, Ferrante M, Van Assche G, Vermeire S (2014) Targeting TNF-α for the treatment of inflammatory bowel disease. Expert Opin Biol Ther 14:75–101.

Blackshaw LA (2014a) TRPs in visceral sensory pathways. Br J Pharmacol. 171(10):2528-2536.

Blackshaw LA (2014b) Transient receptor potential cation channels in visceral sensory pathways. Br J Pharmacol 171:2528–2536.

Blasius AL, Beutler B (2010) Intracellular Toll-like Receptors. Immunity 32:305–315.

Boesmans W, Owsianik G, Tack J, Voets T, Vanden Berghe P (2011) TRP channels in neurogastroenterology: opportunities for therapeutic intervention. Br J Pharmacol 162:18–37.

Bradesi S, Eutamene H, Garcia-Villar R, Fioramonti J, Buéno L (2002) Acute and chronic stress differently affect visceral sensitivity to rectal distension in female rats. Neurogastroenterol Motil 14:75–82.

Brint EK, MacSharry J, Fanning A, Shanahan F, Quigley EMM (2011) Differential expression of toll-like receptors in patients with irritable bowel syndrome. Am J Gastroenterol 106:329–336.

Brusberg M, Arvidsson S, Kang D, Larsson H, Lindström E, Martinez V (2009) CB1 receptors mediate the analgesic effects of cannabinoids on colorectal distension-induced visceral pain in rodents. J Neurosci 29:1554–1564.

Bueno L, Fioramonti J (2002) Visceral perception: inflammatory and non-inflammatory mediators. Gut 51:i19–i23.

Buffie CG, Pamer EG (2013) Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol 13:790–801.

Bulmer DC, Grundy D (2011) Achieving translation in models of visceral pain. Curr Opin Pharmacol 11:575–581.

Camilleri M (2009) Serotonin in the gastrointestinal tract. Curr Opin Endocrinol Diabetes Obes 16:53–59.

Camilleri M, Ford MJ (1998) Review article: colonic sensorimotor physiology in health, and its alteration in constipation and diarrhoeal disorders. Aliment Pharmacol Ther 12:287–302.

Cario E (2008) Therapeutic impact of toll-like receptors on inflammatory bowel diseases: a multiple-edged sword. Inflamm Bowel Dis 14:411–421.

Chang F-Y (2014) Irritable bowel syndrome: the evolution of multi-dimensional looking and multidisciplinary treatments. World J Gastroenterol 20:2499–2514.

Chassard C, Dapoigny M, Scott KP, Crouzet L, Del'homme C, Marquet P, Martin JC, Pickering G, Ardid D, Eschalier A, Dubray C, Flint HJ, Bernalier-Donadille A (2012) Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome. Aliment Pharmacol Ther 35:828–838.

Chen J, Diacovo TG, Grenache DG, Santoro SA, Zutter MM (2002) The alpha(2) integrin subunit-deficient mouse: a multifaceted phenotype including defects of branching morphogenesis and hemostasis. Am J Pathol 161:337–344.

Chow JYC, Li ZJ, Wu WKK, Cho CH (2013) Cathelicidin a potential therapeutic peptide for gastrointestinal inflammation and cancer. World J Gastroenterol 19:2731–2735.

Clark MA, Hirst BH, Jepson MA (1998) M-cell surface beta1 integrin expression and invasin-mediated targeting of Yersinia pseudotuberculosis to mouse Peyer's patch M cells. Infect Immun 66:1237–1243.

Clevers HC, Bevins CL (2013) Paneth cells: maestros of the small intestinal crypts. Annu Rev Physiol 75:289–311.

Collins S, Verdu E, Denou E, Bercik P (2009) The role of pathogenic microbes and commensal bacteria in irritable bowel syndrome. Dig Dis 27 Suppl 1:85–89.

Collins SM (2014) A role for the gut microbiota in IBS. Nat Rev Gastroenterol Hepatol 11:497–505.

Collins SM, Bercik P (2009) The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology 136:2003–2014.

Collins SM, Surette M, Bercik P (2012) The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 10:735–742.

Cremon C, Carini G, Wang B, Vasina V, Cogliandro RF, De Giorgio R, Stanghellini V, Grundy D, Tonini M, De Ponti F, Corinaldesi R, Barbara G (2011) Intestinal serotonin release, sensory neuron activation, and abdominal pain in irritable bowel syndrome. Am J Gastroenterol 106:1290–1298.

Critchley-Thorne RJ, Stagg AJ, Vassaux G (2006) Recombinant Escherichia coli expressing invasin targets the Peyer's patches: the basis for a bacterial formulation for oral vaccination. Mol Ther 14:183–191.

Crosnier C, Stamataki D, Lewis J (2006) Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat Rev Genet 7:349–359.

Croswell A, Amir E, Teggatz P, Barman M, Salzman NH (2009) Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. Infect Immun 77:2741–2753.

Da Re S, Valle J, Charbonnel N, Beloin C, Latour-Lambert P, Faure P, Turlin E, Le Bouguénec C, Renauld-Mongénie G, Forestier C, Ghigo J-M (2013) Identification of commensal Escherichia coli genes involved in biofilm resistance to pathogen colonization. PLoS One 8:e61628.

Dai C, Zheng C-Q, Jiang M, Ma X-Y, Jiang L-J (2013) Probiotics and irritable bowel syndrome. World J Gastroenterol 19:5973–5980.

De Jonge WJ (2013) The Gut's Little Brain in Control of Intestinal Immunity. ISRN Gastroenterol 2013:630159.

De Palma G, Nadal I, Medina M, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y (2010) Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. BMC Microbiol 10:63.

De Petrocellis L, Di Marzo V (2009) Role of endocannabinoids and endovanilloids in Ca2+ signalling. Cell Calcium 45:611–624.

De Petrocellis L, Orlando P, Moriello AS, Aviello G, Stott C, Izzo AA, Di Marzo V (2012) Cannabinoid actions at TRPV channels: effects on TRPV3 and TRPV4 and their potential relevance to gastrointestinal inflammation. Acta Physiol (Oxf) 204:255–266.

Demir IE, Schäfer K-H, Tieftrunk E, Friess H, Ceyhan GO (2013) Neural plasticity in the gastrointestinal tract: chronic inflammation, neurotrophic signals, and hypersensitivity. Acta Neuropathol 125:491–509.

Derrien M, Van Baarlen P, Hooiveld G, Norin E, Müller M, de Vos WM (2011) Modulation of Mucosal Immune Response, Tolerance, and Proliferation in Mice Colonized by the Mucin-Degrader Akkermansia muciniphila. Front Microbiol 2:166.

Derrien M, Vaughan EE, Plugge CM, de Vos WM (2004) Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol 54:1469–1476.

Dinan TG, Cryan JF (2012) Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology 37:1369–1378.

Dinoto A, Suksomcheep A, Ishizuka S, Kimura H, Hanada S, Kamagata Y, Asano K, Tomita F, Yokota A (2006) Modulation of rat cecal microbiota by administration of raffinose and encapsulated Bifidobacterium breve. Appl Environ Microbiol 72:784–792.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638.

Fernández-Blanco JA, Barbosa S, Sánchez de Medina F, Martínez V, Vergara P (2011) Persistent epithelial barrier alterations in a rat model of postinfectious gut dysfunction. Neurogastroenterol Motil 23:e523–533.

Foster JA, McVey Neufeld K-A (2013) Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci 36:305–312.

Fukudo S (2013) Stress and visceral pain: focusing on irritable bowel syndrome. Pain 154 Suppl :S63–70.

Fukushima K, Sasaki I, Ogawa H, Naito H, Funayama Y, Matsuno S (1999) Colonization of microflora in mice: mucosal defense against luminal bacteria. J Gastroenterol 34:54–60.

Fung TC, Artis D, Sonnenberg GF (2014) Anatomical localization of commensal bacteria in immune cell homeostasis and disease. Immunol Rev 260:35–49.

Gaekwad J, Zhang Y, Zhang W, Reeves J, Wolfert MA, Boons G-J (2010) Differential induction of innate immune responses by synthetic lipid a derivatives. J Biol Chem 285:29375–29386.

Gallo RL, Hooper L V (2012) Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 12:503–516.

Geremia A, Biancheri P, Allan P, Corazza GR, Di Sabatino A, Di A (2013) Innate and adaptive immunity in inflammatory bowel disease. Autoimmun Rev 13(1):3-10. .

Gershon MD, Tack J (2007) The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 132:397–414.

Ghia J-E, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT, Côté F, Mallet J, Khan WI (2009) Serotonin has a key role in pathogenesis of experimental colitis. Gastroenterology 137:1649–1660.

Gionchetti P, Rizzello F, Lammers K-M, Morselli C, Sollazzi L, Davies S, Tambasco R, Calabrese C, Campieri M (2006) Antibiotics and probiotics in treatment of inflammatory bowel disease. World J Gastroenterol 12:3306–3313.

Gómez-Llorente C, Muñoz S, Gil A (2010) Role of Toll-like receptors in the development of immunotolerance mediated by probiotics. Proc Nutr Soc 69:381–389.

Grangette C (2012) Bifidobacteria and subsets of dendritic cells: friendly players in immune regulation! Gut 61:331–332.

Gray AC, Coupar IM, White PJ (2006) Comparison of opioid receptor distributions in the rat ileum. Life Sci 78:1610–1616.

Grenham S, Clarke G, Cryan JF, Dinan TG (2011) Brain-gut-microbe communication in health and disease. Front Physiol 2:94.

Gulewitsch MD, Enck P, Schwille-Kiuntke J, Weimer K, Schlarb AA (2013) Mental Strain and Chronic Stress among University Students with Symptoms of Irritable Bowel Syndrome. Gastroenterol Res Pract 2013:206574.

Gutschow M V, Hughey JJ, Ruggero NA, Bajar BT, Valle SD, Covert MW (2013) Single-cell and population NF-κB dynamic responses depend on lipopolysaccharide preparation. PLoS One 8:e53222.

Guyonnet D, Chassany O, Ducrotte P, Picard C, Mouret M, Mercier C-H, Matuchansky C (2007) Effect of a fermented milk containing Bifidobacterium animalis DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: a multicentre, randomized, double-blind, controlled trial. Aliment Pharmacol Ther 26:475–486.

Harmsen HJM, Elfferich P, Schut F, Welling GW (1999) A 16S rRNA-targeted Probe for Detection of Lactobacilli and Enterococci in Faecal Samples by Fluorescent In Situ Hybridization. Microb Ecol Heal Dis 11:3-12.

Hildebrand F, Nguyen TLA, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, Liston A, Raes J (2013) Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol 14:R4.

Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, Kirn TJ, Bushman FD, Artis D (2010) Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol 3:148–158.

Ho S, Pothoulakis C, Koon HW (2013) Antimicrobial peptides and colitis. Curr Pharm Des 19:40–47.

Hoffman EM, Zhang Z, Anderson MB, Schechter R, Miller KE (2011) Potential mechanisms for hypoalgesia induced by anti-nerve growth factor immunoglobulin are identified using autoimmune nerve growth factor deprivation. Neuroscience 193:452–465.

Holschneider DP, Bradesi S, Mayer EA (2011) The role of experimental models in developing new treatments for irritable bowel syndrome. Expert Rev Gastroenterol Hepatol 5:43–57.

Holzer P (2008) TRPV1: a new target for treatment of visceral pain in IBS? Gut 57:882–884.

Holzer P (2009) Opioid receptors in the gastrointestinal tract. Regul Pept 155:11–17.

Hong S, Fan J, Kemmerer ES, Evans S, Li Y, Wiley JW (2009) Reciprocal changes in vanilloid (TRPV1) and endocannabinoid (CB1) receptors contribute to visceral hyperalgesia in the water avoidance stressed rat. Gut 58:202–210.

Hughes P a, Zola H, Penttila IA, Blackshaw LA, Andrews JM, Krumbiegel D (2013) Immune activation in irritable bowel syndrome: can neuroimmune interactions explain symptoms? Am J Gastroenterol 108:1066–1074.

Hungin APS, Mulligan C, Pot B, Whorwell P, Agréus L, Fracasso P, Lionis C, Mendive J, Philippart de Foy J-M, Rubin G, Winchester C, de Wit N (2013) Systematic review: probiotics in the management of lower gastrointestinal symptoms in clinical practice -- an evidence-based international guide. Aliment Pharmacol Ther 38:864–886.

Hutchinson MR, Lewis SS, Coats BD, Skyba DA, Crysdale NY, Berkelhammer DL, Brzeski A, Northcutt A, Vietz CM, Judd CM, Maier SF, Watkins LR, Johnson KW (2009) Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). Brain Behav Immun 23:240–250.

Ivanov II, Honda K (2012) Intestinal commensal microbes as immune modulators. Cell Host Microbe 12:496–508.

Izzo AA, Sharkey KA (2010) Cannabinoids and the gut: new developments and emerging concepts. Pharmacol Ther 126:21–38.

Jalanka-Tuovinen J, Salojärvi J, Salonen A, Immonen O, Garsed K, Kelly FM, Zaitoun A, Palva A, Spiller RC, de Vos WM (2013) Faecal microbiota composition and host-microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome. Gut. doi: 10.1136/gutjnl-2013-305994.

Jardí F, Fernández-Blanco JA, Martínez V, Vergara P (2014) Plasticity of Dorsal Root Ganglion Neurons in a Rat Model of Post-Infectious Gut Dysfunction: Potential Implication of Nerve Growth Factor. Scand J Gastroenterol (in press).

Johansson ME V (2014) Mucus Layers in Inflammatory Bowel Disease. Inflamm Bowel Dis. (in press)

Johansson ME V, Larsson JMH, Hansson GC (2011) The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A 108 Suppl :4659–4665.

Jones RCW, Gebhart GF (2004) Models of visceral pain: colorectal distension (CRD). Curr Protoc Pharmacol Chapter 5:Unit 5.36.

Julio-Pieper M, O'Mahony CM, Clarke G, Bravo JA, Dinan TG, Cryan JF (2012) Chronic stress-induced alterations in mouse colonic 5-HT and defecation responses are strain dependent. Stress 15:218–226.

Kamada N, Chen GY, Inohara N, Núñez G (2013) Control of pathogens and pathobionts by the gut microbiota. Nat Immunol 14:685–690.

Kamada N, Núñez G (2014) Regulation of the immune system by the resident intestinal bacteria. Gastroenterology 146:1477–1488.

Kamdar K, Nguyen V, DePaolo RW (2013) Toll-like receptor signaling and regulation of intestinal immunity. Virulence 4:207–212.

Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E (2005) Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest 115:695–702.

Kennedy PJ, Cryan JF, Quigley EMM, Dinan TG, Clarke G (2014) A sustained hypothalamic-pituitary-adrenal axis response to acute psychosocial stress in irritable bowel syndrome. Psychol Med 44:3123–3134.

Khan S, Chang L (2010) Diagnosis and management of IBS. Nat Rev Gastroenterol Hepatol 7:565–581.

Kidd M, Gustafsson BI, Drozdov I, Modlin IM (2009) IL1beta- and LPS-induced serotonin secretion is increased in EC cells derived from Crohn's disease. Neurogastroenterol Motil 21:439–450.

Kim YS, Ho SB (2010) Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 12:319–330.

Kobayashi T, Matsuoka K, Sheikh SZ, Elloumi HZ, Kamada N, Hisamatsu T, Hansen JJ, Doty KR, Pope SD, Smale ST, Hibi T, Rothman PB, Kashiwada M, Plevy SE (2011) NFIL3 is a regulator of IL-12 p40 in macrophages and mucosal immunity. J Immunol 186:4649–4655.

Kraneveld AD, Rijnierse A, Nijkamp FP, Garssen J (2008) Neuro-immune interactions in inflammatory bowel disease and irritable bowel syndrome: future therapeutic targets. Eur J Pharmacol 585:361–374.

Krimi RB, Kotelevets L, Dubuquoy L, Plaisancié P, Walker F, Lehy T, Desreumaux P, Van Seuningen I, Chastre E, Forgue-Lafitte M-E, Marie J-C (2008) Resistin-like molecule beta regulates intestinal mucous secretion and curtails TNBS-induced colitis in mice. Inflamm Bowel Dis 14:931–941.

Kuiken SD, Tytgat GN, Boeckxstaens GE (2005) Review article: drugs interfering with visceral sensitivity for the treatment of functional gastrointestinal disorders--the clinical evidence. Aliment Pharmacol Ther 21:633–651.

Kuner R (2010) Central mechanisms of pathological pain. Nat Med 16:1258–1266.

Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F (2001) A new model of visceral pain and referred hyperalgesia in the mouse. Pain 92:335–342.

Larauche M, Mulak A, Kim YS, Labus J, Million M, Taché Y (2012) Visceral analgesia induced by acute and repeated water avoidance stress in rats: sex difference in opioid involvement. Neurogastroenterol Motil 24:1031–e547.

Larauche M, Mulak A, Taché Y (2011) Stress-related alterations of visceral sensation: animal models for irritable bowel syndrome study. J Neurogastroenterol Motil 17:213–234.

Lavelle EC, Murphy C, O'Neill LAJ, Creagh EM (2010) The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. Mucosal Immunol 3:17–28.

Lee J, Chuang T-H, Redecke V, She L, Pitha PM, Carson DA, Raz E, Cottam HB (2003) Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. Proc Natl Acad Sci U S A 100:6646–6651.

Lee KN, Lee OY (2014) Intestinal microbiota in pathophysiology and management of irritable bowel syndrome. World J Gastroenterol 20:8886–8897.

Leulier F, Lemaitre B (2008) Toll-like receptors--taking an evolutionary approach. Nat Rev Genet 9:165–178.

Lewin GR, Lechner SG, Smith ESJ (2014) Nerve growth factor and nociception: from experimental embryology to new analgesic therapy. Handb Exp Pharmacol 220:251–282.

Lindén SK, Bierne H, Sabet C, Png CW, Florin TH, McGuckin MA, Cossart P (2008) Listeria monocytogenes internalins bind to the human intestinal mucin MUC2. Arch Microbiol 190:101–104.

Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC (2006) Functional bowel disorders. Gastroenterology 130:1480–1491.

Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A (2013) Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathog 5:23.

Ludigs K, Parfenov V, Du Pasquier RA, Guarda G (2012) Type I IFN-mediated regulation of IL-1 production in inflammatory disorders. Cell Mol Life Sci 69:3395–3418.

Lutgendorff F, Akkermans LMA, Söderholm JD (2008) The role of microbiota and probiotics in stress-induced gastro-intestinal damage. Curr Mol Med 8:282–298.

Mantani Y, Nishida M, Yuasa H, Yamamoto K, Takahara E-I, Omotehara T, Udayanga KGS, Kawano J, Yokoyama T, Hoshi N, Kitagawa H (2014) Ultrastructural and histochemical study on the Paneth cells in the rat ascending colon. Anat Rec (Hoboken) 297:1462–1471.

Margolis KG, Stevanovic K, Li Z, Yang QM, Oravecz T, Zambrowicz B, Jhaver KG, Diacou A, Gershon MD (2014) Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine. Gut 63:928–937.

Martínez V, Thakur S, Mogil JS, Taché Y, Mayer E a (1999) Differential effects of chemical and mechanical colonic irritation on behavioral pain response to intraperitoneal acetic acid in mice. Pain 81:179–186.

Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, Darfeuille-Michaud A, Barnich N (2014) Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 63:116–124.

Maruya M, Kawamoto S, Kato LM, Fagarasan S (2013) Impaired selection of IgA and intestinal dysbiosis associated with PD-1-deficiency. Gut Microbes 4:165–171.

Matsuo K, Ota H, Akamatsu T, Sugiyama A, Katsuyama T (1997) Histochemistry of the surface mucous gel layer of the human colon. Gut 40:782–789.

Maynard CL, Weaver CT (2008) Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. Immunol Rev 226:219–233.

McKernan DP, Gaszner G, Quigley EM, Cryan JF, Dinan TG (2011) Altered peripheral toll-like receptor responses in the irritable bowel syndrome. Aliment Pharmacol Ther 33:1045–1052.

Melgar S, Shanahan F (2010) Inflammatory bowel disease—from mechanisms to treatment strategies. Autoimmunity 43:463–477.

Meseguer V et al. (2014) TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. Nat Commun 5:3125.

Montiel-Castro AJ, González-Cervantes RM, Bravo-Ruiseco G, Pacheco-López G (2013) The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. Front Integr Neurosci 7:70.

Moran GW, Leslie FC, Levison SE, Worthington J, McLaughlin JT (2008) Enteroendocrine cells: neglected players in gastrointestinal disorders? Therap Adv Gastroenterol 1:51–60.

Munford RS (2008) Sensing gram-negative bacterial lipopolysaccharides: a human disease determinant? Infect Immun 76:454–465.

Muniz LR, Knosp C, Yeretssian G (2012) Intestinal antimicrobial peptides during homeostasis, infection, and disease. Front Immunol 3:310.

Nilius B, Mahieu F (2006) A road map for TR(I)Ps. Mol Cell 22:297–307.

O'Mahony SM, Felice VD, Nally K, Savignac HM, Claesson MJ, Scully P, Woznicki J, Hyland NP, Shanahan F, Quigley EM, Marchesi JR, O'Toole PW, Dinan TG, Cryan JF (2014) Disturbance of the Gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. Neuroscience 277C:885–901.

Ohman L, Simrén M (2010) Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. Nat Rev Gastroenterol Hepatol 7:163–173.

Ordás I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ (2012) Ulcerative colitis. Lancet 380:1606–1619.

Ostaff MJ, Stange EF, Wehkamp J (2013) Antimicrobial peptides and gut microbiota in homeostasis and pathology. EMBO Mol Med 5:1465–1483.

Pabst O (2012) New concepts in the generation and functions of IgA. Nat Rev Immunol 12:821–832.

Parkes GC, Brostoff J, Whelan K, Sanderson JD (2008) Gastrointestinal microbiota in irritable bowel syndrome: their role in its pathogenesis and treatment. Am J Gastroenterol 103:1557–1567.

Paul G, Khare V, Gasche C (2012) Inflamed gut mucosa: downstream of interleukin-10. Eur J Clin Invest 42:95–109.

Peluso I, Pallone F, Monteleone G (2006) Interleukin-12 and Th1 immune response in Crohn's disease: pathogenetic relevance and therapeutic implication. World J Gastroenterol 12:5606–5610.

Peterson LW, Artis D (2014) Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol 14:141–153.

Petrella C, Agostini S, Alema' GS, Casolini P, Carpino F, Giuli C, Improta G, Linari G, Petrozza V, Broccardo M (2010) Cannabinoid agonist WIN55,212 in vitro inhibits interleukin-6 (IL-6) and monocyte chemo-attractant protein-1 (MCP-1) release by rat pancreatic acini and in vivo induces dual effects on the course of acute pancreatitis. Neurogastroenterol Motil 22:1248–56, e323.

Philippe D, Chakass D, Thuru X, Zerbib P, Tsicopoulos A, Geboes K, Bulois P, Breisse M, Vorng H, Gay J, Colombel J-F, Desreumaux P, Chamaillard M (2006) Mu opioid receptor expression is increased in inflammatory bowel diseases: implications for homeostatic intestinal inflammation. Gut 55:815–823.

Phillis BD, Martin CM, Kang D, Larsson H, Lindström EA, Martinez V, Blackshaw LA (2009) Role of TRPV1 in high-threshold rat colonic splanchnic afferents is revealed by inflammation. Neurosci Lett 459:57–61.

Piche T (2014) Tight junctions and IBS--the link between epithelial permeability, low-grade inflammation, and symptom generation? Neurogastroenterol Motil 26:296–302.

Pol O, Alameda F, Puig MM (2001) Inflammation enhances mu-opioid receptor transcription and expression in mice intestine. Mol Pharmacol 60:894–899.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085–2088.

Posserud I, Agerforz P, Ekman R, Björnsson ES, Abrahamsson H, Simrén M (2004) Altered visceral perceptual and neuroendocrine response in patients with irritable bowel syndrome during mental stress. Gut 53:1102–1108.

Qin J et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65.

Quigley EMM (2011) Therapies aimed at the gut microbiota and inflammation: antibiotics, prebiotics, probiotics, synbiotics, anti-inflammatory therapies. Gastroenterol Clin North Am 40:207–222.

Rajilić-Stojanović M, Biagi E, Heilig HGHJ, Kajander K, Kekkonen RA, Tims S, de Vos WM (2011) Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 141:1792–1801.

Ratcliffe EM (2011) Molecular development of the extrinsic sensory innervation of the gastrointestinal tract. Auton Neurosci 161:1–5.

Reigstad CS, Kashyap PC (2013) Beyond phylotyping: understanding the impact of gut microbiota on host biology. Neurogastroenterol Motil 25:358–372.

Rescigno M (2011) The intestinal epithelial barrier in the control of homeostasis and immunity. Trends Immunol 32:256–264.

Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6:306–314.

Ringel Y, Williams RE, Kalilani L, Cook SF (2009) Prevalence, characteristics, and impact of bloating symptoms in patients with irritable bowel syndrome. Clin Gastroenterol Hepatol 7:68–72.

Rodríguez-Fandiño O, Hernández-Ruiz J, Schmulson M (2010) From cytokines to toll-like receptors and beyond - current knowledge and future research needs in irritable bowel syndrome. J Neurogastroenterol Motil 16:363–373.

Round JL, Mazmanian SK (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A 107:12204–12209.

Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel J-F, Ardid D, Desreumaux P (2007) Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 13:35–37.

Sainathan SK, Bishnupuri KS, Aden K, Luo Q, Houchen CW, Anant S, Dieckgraefe BK (2012) Toll-like receptor-7 ligand Imiquimod induces type I interferon and antimicrobial peptides to ameliorate dextran sodium sulfate-induced acute colitis. Inflamm Bowel Dis 18:955–967.

Saito K, Katakura K, Suzuki R, Suzuki T, Ohira H (2013) Modulating Toll-like receptor 4 signaling pathway protects mice from experimental colitis. Fukushima J Med Sci 59:81–88.

Salzman NH, Bevins CL (2013) Dysbiosis—A consequence of Paneth cell dysfunction. Semin Immunol 25:334–341.

Salzman NH, Underwood M a, Bevins CL (2007) Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. Semin Immunol 19:70–83.

Sanders KM (2008) Regulation of smooth muscle excitation and contraction. Neurogastroenterol Motil 20 Suppl 1:39–53.

Sarna SK (2010) Colonic Motility: From Bench Side to Bedside [2010] - PubMed - NCBI. San Rafael Morgan Claypool Life Sci Available at: http://www.ncbi.nlm.nih.gov/pubmed/21452445.

Sartor RB, Mazmanian SK (2012) Intestinal Microbes in Inflammatory Bowel Diseases. Am J Gastroenterol Suppl 1:15–21.

Sauer R-S, Hackel D, Morschel L, Sahlbach H, Wang Y, Mousa SA, Roewer N, Brack A, Rittner HL (2014) Toll like receptor (TLR)-4 as a regulator of peripheral endogenous opioid-mediated analgesia in inflammation. Mol Pain 10:10.

Saulnier DM, Ringel Y, Heyman MB, Foster JA, Bercik P, Shulman RJ, Versalovic J, Verdu EF, Dinan TG, Hecht G, Guarner F (2013) The intestinal microbiome, probiotics and prebiotics in neurogastroenterology. Gut Microbes 4:17–27.

Schwab C, Berry D, Rauch I, Rennisch I, Ramesmayer J, Hainzl E, Heider S, Decker T, Kenner L, Müller M, Strobl B, Wagner M, Schleper C, Loy A, Urich T (2014) Longitudinal study of murine microbiota activity and interactions with the host during acute inflammation and recovery. ISME J 8(5):1101-1114.

Sekirov I, Russell SL, Antunes LCM, Finlay BB (2010) Gut microbiota in health and disease. Physiol Rev 90:859–904.

Shah N, Kammermeier J, Elawad M, Glocker E-O (2012) Interleukin-10 and interleukin-10-receptor defects in inflammatory bowel disease. Curr Allergy Asthma Rep 12:373–379.

Sharma R, Schumacher U, Ronaasen V, Coates M (1995) Rat intestinal mucosal responses to a microbial flora and different diets. Gut 36:209–214.

Shibolet O, Podolsky DK (2007) TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. Am J Physiol Gastrointest Liver Physiol 292:G1469–1473.

Sikandar S, Dickenson AH (2012) Visceral pain: the ins and outs, the ups and downs. Curr Opin Support Palliat Care 6:17–26.

Simrén M, Barbara G, Flint HJ, Spiegel BMR, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG (2013) Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut 62:159–176.

Sommer F, Bäckhed F (2013) The gut microbiota--masters of host development and physiology. Nat Rev Microbiol 11:227–238.

Spencer NJ (2001) Control of migrating motor activity in the colon. Curr Opin Pharmacol 1:604–610.

Spiller R, Aziz Q, Creed F, Emmanuel A, Houghton L, Hungin P, Jones R, Kumar D, Rubin G, Trudgill N, Whorwell P (2007) Guidelines on the irritable bowel syndrome: mechanisms and practical management. Gut 56:1770–1798.

Spiller R, Lam C (2011) The shifting interface between IBS and IBD. Curr Opin Pharmacol 11:586–592.

Stanzel RDP, Lourenssen S, Blennerhassett MG (2008) Inflammation causes expression of NGF in epithelial cells of the rat colon. Exp Neurol 211:203–213.

Storr MA, Yüce B, Andrews CN, Sharkey KA (2008) The role of the endocannabinoid system in the pathophysiology and treatment of irritable bowel syndrome. Neurogastroenterol Motil 20:857–868.

Strugnell RA, Wijburg OLC (2010) The role of secretory antibodies in infection immunity. Nat Rev Microbiol 8:656–667.

Swidsinski A, Loening-Baucke V, Lochs H, Hale L-P (2005) Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World J Gastroenterol 11:1131–1140.

Taché Y, Martinez V, Wang L, Million M (2004) CRF1 receptor signaling pathways are involved in stress-related alterations of colonic function and viscerosensitivity: implications for irritable bowel syndrome. Br J Pharmacol 141:1321–1330.

Terán-Ventura E, Aguilera M, Vergara P, Martínez V (2014) Specific changes of gut commensal microbiota and TLRs during indomethacin-induced acute intestinal inflammation in rats. J Crohns Colitis 8(9):1043-1054.

Terán-Ventura E, Roca M, Martin MT, Abarca ML, Martinez V, Vergara P (2010) Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats. Microb Ecol 60:691–702.

Theodorou V, Belgnaoui AA, Agostini S, Eutamene H (2014) Effect of commensals and probiotics on visceral sensitivity and pain in irritable bowel syndrome. Gut Microbes 5(3).

Tramullas M, Finger BC, Moloney RD, Golubeva A V, Moloney G, Dinan TG, Cryan JF (2014) Toll-like receptor 4 regulates chronic stress-induced visceral pain in mice. Biol Psychiatry 76:340–348.

Treuting PM, Dintzis SM (2012) Comparative Anatomy and Histology. Elsevier. Chapter 12, p177-192.

Tsang SW, Zhao M, Wu J, Sung JJY, Bian Z-X (2012) Nerve growth factor-mediated neuronal plasticity in spinal cord contributes to neonatal maternal separation-induced visceral hypersensitivity in rats. Eur J Pain 16:463–472.

Tschurtschenthaler M, Wang J, Fricke C, Fritz TMJ, Niederreiter L, Adolph TE, Sarcevic E, Künzel S, Offner FA, Kalinke U, Baines JF, Tilg H, Kaser A (2014) Type I interferon signalling in the intestinal epithelium affects Paneth cells, microbial ecology and epithelial regeneration. Gut. doi: 10.1136/gutjnl-2013-305863.

Turroni F, Ventura M, Buttó LF, Duranti S, O'Toole PW, Motherway MO, van Sinderen D (2014) Molecular dialogue between the human gut microbiota and the host: a Lactobacillus and Bifidobacterium perspective. Cell Mol Life Sci 71:183–203.

Ueda T, Yamada T, Ugawa S, Ishida Y, Shimada S (2009) TRPV3, a thermosensitive channel is expressed in mouse distal colon epithelium. Biochem Biophys Res Commun 383:130–134.

Uematsu S, Fujimoto K (2010) The innate immune system in the intestine. Microbiol Immunol 54:645–657.

Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, Ley R, Wakeland EK, Hooper L V (2011) The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science 334:255–258.

Van der Waaij LA, Kroese FGM, Visser A, Nelis GF, Westerveld BD, Jansen PLM, Hunter JO (2004) Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. Eur J Gastroenterol Hepatol 16:669–674.

Van der Waaij LA, Limburg PC, Mesander G, van der Waaij D (1996) In vivo IgA coating of anaerobic bacteria in human faeces. Gut 38:348–354.

Venkatachalam K, Montell C (2007) TRP channels. Annu Rev Biochem 76:387–417.

Verdú EF, Bercik P, Verma-Gandhu M, Huang X-X, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM (2006) Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 55:182–190.

Vergnolle N (2014) TRPV4: new therapeutic target for inflammatory bowel diseases. Biochem Pharmacol 89:157–161.

Vicario M, Alonso C, Guilarte M, Serra J, Martínez C, González-Castro AM, Lobo B, Antolín M, Andreu AL, García-Arumí E, Casellas M, Saperas E, Malagelada JR, Azpiroz F, Santos J (2012) Chronic psychosocial stress induces reversible mitochondrial damage and corticotropin-releasing factor receptor type-1 upregulation in the rat intestine and IBS-like gut dysfunction. Psychoneuroendocrinology 37:65–77.

Vijay-Kumar M, Aitken JD, Gewirtz AT (2008) Toll like receptor-5: protecting the gut from enteric microbes. Semin Immunopathol 30:11–21.

Waldner MJ, Neurath MF (2014) Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development. Semin Immunol 26(1):75-9..

Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, Russell SL, Vallance BA, Finlay BB (2011) Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. Infect Immun 79:1536–1545.

Wright K, Rooney N, Feeney M, Tate J, Robertson D, Welham M, Ward S (2005) Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. Gastroenterology 129:437–453.

Wright KL, Duncan M, Sharkey KA (2008) Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation. Br J Pharmacol 153:263–270.

Yu LC-H, Shih Y-A, Wu L-L, Lin Y-D, Kuo W-T, Peng W-H, Lu K-S, Wei S-C, Ni Y-H (2014) Enteric dysbiosis promotes antibiotic-resistant bacterial infection: systemic dissemination of resistant and commensal bacteria through epithelial transcytosis. Am J Physiol Gastrointest Liver Physiol. pii: ajpgi.00070.2014.

Yue C, Ma B, Zhao Y, Li Q, Li J (2012) Lipopolysaccharide-induced bacterial translocation is intestine site-specific and associates with intestinal mucosal inflammation. Inflammation 35:1880–1888.

Zoppi S, Madrigal JLM, Pérez-Nievas BG, Marín-Jiménez I, Caso JR, Alou L, García-Bueno B, Colón A, Manzanares J, Gómez-Lus ML, Menchén L, Leza JC (2012) Endogenous cannabinoid system regulates intestinal barrier function in vivo through cannabinoid type 1 receptor activation. Am J Physiol Gastrointest Liver Physiol 302:G565–71.

APENDICCES

PUBLICATIONS DERIVED FROM THIS THESIS

Papers:

Aguilera M, Vergara P, Martínez V. Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. *Neurogastroenterol Motil. 2013; 25(8): e515-29.*

Aguilera M, Vergara P, Martínez V. Environment-related adaptive changes of gut commensal microbiota do not alter colonic toll-like receptors but modulate the local expression of sensory-related systems in rats. *Microb Ecol. 2013; 66(1): 232-43.*

Aguilera M, Cerdà-Cuéllar M, Martínez V. Antibiotic-induced dysbiosis alters host-bacterial interactions and leads to colonic sensory and motor changes in mice. *Gut Microbes* (Submitted).

Abstracts:

Aguilera M, Vergara P, Martínez V. Antibiotic-induced dysbiosis of the gut commensal microbiota does not affect the endocrine and colonic motor responses to stress in mice. *Gastroenterology* 2011; 140 (Sppl 1): S-372.
Digestive Disease Week (DDW). Chicago (USA), 2011. Poster

Aguilera M, Estévez J, Vergara P, Martinez V. Stress- and antibiotic-induced dysbiosis of gut commensal microbiota (GCM) disrupts the mucus layer and promotes bacterial wall adherence in mice.
15th International Congress of Mucosal Immunology. Paris (France), 2011. Poster

Aguilera M, Estévez J, Vergara P, Martinez V. Stimulation of colonic toll-like receptor 7 induces a local immune response without histological signs of inflammation or changes in bacterial wall adherence in rats. *Gastroenterology* 2012; 142 (Sppl 1): S-677.
Digestive Disease Week (DDW). San Diego (USA), 2012. Poster

Aguilera M, Vergara P, Martinez V. Environment-related adaptive changes of gut commensal microbiota do not alter colonic toll-like-dependent bacterial recognition systems but modulate the local expression of sensory-related systems. *Gastroenterology* 2012; 142 (Sppl 1): S-676.
Digestive Disease Week (DDW). San Diego (USA), 2012. Poster

Aguilera M, Vergara P, Martinez V. Stress- and antibiotic-induced colonic dysbiosis results in increased bacterial wall adherence and enhanced local expression of visceral sensory-related systems in mice. *Gastroenterology* 2012; 142 (Sppl 1): S-555.
Digestive Disease Week (DDW). San Diego (USA), 2012. Poster

Aguilera M, Estévez J, Vergara P, Martínez V. Stimulation of colonic Toll-like receptors induces a local immune response without histological signs of inflammation or changes in bacterial wall adherence in rats. *Neurogastroenterol Motil* 2012; 24 (Sppl 2): 43–190
International Neurogastroenterology and Motility Meeting. Bologna (Italy), 2012. Poster

Aguilera M, Estévez J, Martínez V. LPS-dependent stimulation of colonic Toll-like receptors (TLRs) enhances the intestinal endocannabinoid system in the absence of inflammatory-like responses or changes in bacterial wall adherence in rats. *Gut* 2012; 61 (S3): A175.
United European Gastroenterology Week (UEGW). Amsterdam (Holland), 2012. Poster

Aguilera M, Vergara P, Martínez V. Visceral pain-related responses are attenuated in mice with antibiotic-induced intestinal dysbiosis. *Gastroenterology* 2013; 144 (Sppl 1): S-933.
Digestive Disease Week (DDW). Orlando (USA), 2013. Poster

Aguilera M, Pla J, Martínez V. Stimulation of colonic Toll-like receptor 4 (TLR4) with LPS enhances host-bacterial interactions and leads to a local immune activation and an up-regulation of sensory-related systems in rats. *Gastroenterology* 2014; 144 (Sppl 1): S-831.
Digestive Disease Week (DDW). Orlando (USA), 2013. Poster

Aguilera M, Vergara P, Martínez V. Antibiotic-induced intestinal dysbiosis attenuated visceral pain-related responses in mice. *United European Gastroenterology Journal* 2013; 1 (Sppl 1): A240.
United European Gastroenterology Week (UEGW).Berlin (Germany), 2013. Poster

Aguilera M, Pla J, Martínez V. Stimulation of colonic TLR4- and TLR7-mediated bacterial-host interaction systems leads to a distinct local immune activation in rats. *Gastroenterology* 2014; 146 (Sppl 1): S-290.
Digestive Disease Week (DDW). Chicago (USA), 2014. Poster

Aguilera M, Martínez V. Dysbiosis of the gut commensal microbiota modulates neuro-immune sensory systems and visceral nociception in mice. *Acta Physiologica* 2014; 212 (Sppl 698): 27.
XXXVII Congreso de la Sociedad Española de Ciencias Fisiológicas. Granada (Spain), 214.
Oral communication

Aguilera M, Martínez V. Stimulation of colonic Toll-like receptors 4 and 7 induces a specific neuro-immune activation in rats. *Acta Physiologica* 2014; 2012 (Sppl 698): 42.
XXXVII Congreso de la Sociedad Española de Ciencias Fisiológicas. Granada (Spain), 214.
Oral communication

CURRICULUM VITAE

Mònica Aguilera Pujabet
18/02/1986
monica.aguilerap@gmail.com

EDUCATION

2009: Doctor in veterinary Medicine (DVM). Universitat Autònoma de Barcelona, Bellaterra, Spain

2010: Master in Neuroscience. Universitat Autònoma de Barcelona, Bellaterra, Spain

2010: Training course for research staff user of animals for experimental and other scientific purposes – FELASA C Category. Universitat Autònoma de Barcelona, Bellaterra, Spain

In progress: Master in Science and Welfare of laboratory animals – FELASA D Category. Universitat Autònoma de Barcelona, Bellaterra, Spain

GRANTS AND AWARDS

- Research support grant from Dept. of Cell Biology, Physiology and Immunology, Veterinary School, Universitat Autònoma de Barcelona.
- PhD grant: F.P.I. from the Spanish Government (MICINN BES-2010-037699) linked to the project: BFU2009-08229. 2010 - 2014
- Travel award for young Scientists - 18th United European Gastroenterology Week. Barcelona. 2010
- Travel award for young Scientists - 20th United European Gastroenterology Week. Amsterdam. 2012
- Personal Grant – Research abroad (May – August 2013) from Spanish Ministerio de Economía y Competitividad. Alimentary Pharmabiotic Centre, University College Cork

TEACHING EXPERIENCE

- Practical classes (lab assistant) of Physiology. Veterinary, Biomedicine, and Microbiology degrees.
- Practical classes (post-graduate courses–Felasa Category C and D)
- Collaborator: Argo Program (approaching science to high school students)

PRESENT POSITION

R&D specialist at Almirall S.A. (From September 2014)

PUBLICATIONS

Aguilera M, Vergara P, Martínez V. Environment-related adaptive changes of gut commensal microbiota do not alter colonic toll-like receptors but modulate the local expression of sensory-related systems in rats. Microb Ecol. 2013 Jul; 66(1):232-43.

Aguilera M, Vergara P, Martínez V. Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. Neurogastroenterol Motil. 2013 Aug; 25(8):e515-29.

Terán-Ventura E, **Aguilera M**, Vergara P, Martínez V. Specific changes of gut commensal microbiota and TLRs during indomethacin-induced acute intestinal inflammation in rats. J Crohns Colitis. 2014 Feb 21. pii: S1873-9946(14)00049-X. doi: 10.1016/j.crohns.2014.02.001.

Aguilera M, Darby T, Melgar S. The complex role of inflammasomes in the pathogenesis of Inflammatory Bowel Diseases - lessons learned from experimental models. Cytokine Growth Factor Rev. 2014 Apr 18. pii: S1359-6101(14)00036-7. doi: 10.1016/j.cytogfr.2014.04.003.

Barbosa S, **Aguilera M**, Ferrer-Clotas M, Jardí F, Vergara P. Breeding origin in rats determines gut commensal microbiota and intestinal immune expression. Microbial Ecology. Submitted July 2014.

Aguilera M, Vergara P, Martínez V. Antibiotic-induced dysbiosis alters host-bacterial interactions and leads to colonic sensory and motor changes in mice. Gut Microbes. Submitted August 2014

ABSTRACTS AND MEETING PRESENTATIONS

29 abstracts published from 2009

2 National meetings
12 International meetings

Neurogastroenterology & Motility

Neurogastroenterol Motil (2013) **25,** e515–e529 doi: 10.1111/nmo.12154

Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice

M. AGUILERA,*,† P. VERGARA*,†,‡ & V. MARTÍNEZ*,†,‡

*Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain
†Neuroscience Institute, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain
‡Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Madrid, Spain

Abstract

Background *Stress leads to altered gastrointestinal neuro-immune responses. We characterized the interaction between stress and gut commensal microbiota and their role modulating colonic responses to stress, the induction of inflammation, the expression of sensory-related markers, and visceral sensitivity.* ***Methods*** *C57BL/6N female mice were treated (7 days, PO) with non-absorbable-broad spectrum antibiotics (bacitracin/neomycin, 0.4 mg per mouse per day). Simultaneously, mice were subjected to a 1 h per day (7 days) session of psychological stress (water avoidance stress, WAS). Luminal and wall-adhered microbiota were characterized by fluorescent* in situ *hybridization. Cannabinoid receptors 1 and 2 (CB1/2), tryptophan hydroxylase 1 and 2 (TPH1/2), and inflammatory markers were quantified by reverse transcription–quantitative real-time PCR (RT-qPCR) and secretory-IgA (s-IgA) by ELISA. Visceral sensitivity was assessed after the intracolonic administration of capsaicin.* ***Key Results*** *Antibiotics did not affect the defecatory and endocrine responses to stress. However, antibiotics diminished by 2.5-folds total bacterial counts, induced a specific dysbiosis and favored bacterial wall adherence. Combining antibiotics and stress resulted in further reductions in bacterial counts and a dysbiosis, with enhanced bacterial wall adherence. Luminal s-IgA levels increased in dysbiotic mice. Nevertheless, no alterations consistent with the induction of colonic inflammation were observed. Dysbiosis upregulated CB2 expression and stress upregulated CB2 and TPH1 expression. Stress enhanced visceral pain-related responses, an effect prevented by antibiotic treatment.* ***Conclusions & Inferences*** *Manipulations of the commensal microbiota and the interaction host–microbiota are able to modulate the local expression of neuro–immune–endocrine systems within the colon, leading to a modulation of visceral sensitivity. These mechanisms might contribute to the pathogenic and protective roles of microbiota in gastrointestinal homeostasis.*

Keywords *endocannabinoid system, gut commensal microbiota, intestinal dysbiosis, secretory-IgA, serotonergic system, visceral pain.*

Abbreviations: 5-HT, serotonin; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; DAPI, 4′,6-diamidino-2-phenylindole; EC, enterochromaffin cells; FISH, fluorescent *in situ* hybridization; IBS, irritable bowel syndrome; IL-6, interleukin 6; MMCP-I, mouse mast cell protease I; s-IgA, secretory immunoglobulin A; RT-qPCR, reverse transcription–quantitative real-time PCR; TNFα, tumor necrosis factor α; TPH1, tryptophan hydroxylase isoform 1; TPH2, tryptophan hydroxylase isoform 2; WAS, water avoidance stress.

Functional gastrointestinal disorders, represented mainly by irritable bowel syndrome (IBS), are among the most prevalent gastrointestinal alterations in the western population. Alterations in bowel habits, abdominal pain, and discomfort, believed to reflect increased visceral sensitivity, are hallmarks of IBS.[1] Symptoms in IBS fluctuate over time in intensity and character, but the mechanisms underlying these cycles remain unclear. Several factors, including stress, intestinal infection, drugs, and diet have been reported to

Address for Correspondence
Vicente Martínez, Edifici V, Unitat de Fisiologia, Universitat Autònoma de Barcelona, Bellaterra, Barcelona 08193, Spain.
Tel: +34 93 581 3834; fax: +34 93 581 2006;
e-mail: vicente.martinez@uab.es
Received: 17 December 2012
Accepted for publication: 19 April 2013

© 2013 John Wiley & Sons Ltd

exacerbate symptomatology, and might be key components of the pathophysiology of the disease.[2,3] A growing body of evidence suggests that IBS pathogenesis is likely dependent on the interaction between local immune reactions within the intestinal wall and environmental factors in genetically susceptible individuals. In particular, stress and perturbations of the gut commensal microbiota have been recognized as two potential factors contributing to the onset, maintenance, and exacerbation of both functional and inflammatory gastrointestinal disorders.[4,5] Indeed, stressful life events or depression are risk factors for the onset or relapse of intestinal inflammation and for symptoms presentation in IBS patients. Similarly, growing evidences suggest that IBS patients have a dysbiotic intestinal microbiota.[4,6] Despite these evidences, the exact role of gut microbiota and stress, individually or as interactive factors, in the pathophysiology of IBS remains largely unknown.

In this study, we characterized the interaction between stress and microbiota and their potential role modulating functional colonic responses to stress and the induction of inflammatory-like changes in mice. First, we assessed the effects of repetitive psychological stress (water avoidance stress, WAS) and antibiotic treatment, individually or in combination, on the composition of ceco-colonic commensal microbiota and the induction of inflammatory-like changes in the colon. In the same animals, endocrine and colonic motor responses to stress were assessed simultaneously. To characterize the ceco-colonic microbiota, we determined changes in both luminal and wall (epithelium)-adhered microbiota. The assessment of inflammatory responses was based on inflammatory markers, histological evaluation of the colon, and quantification of luminal secretory-IgA (s-IgA). s-IgA is considered the main anti-inflammatory immunoglobulin of the mucosal intestinal immune system regulating the number, composition, and functions of luminal bacteria.[7,8] Moreover, we also determined changes in relevant systems that have been involved in sensory responses within the colon, with particular relevance to IBS, namely the endocannabinoid and the serotonergic systems. For this, colonic expression of cannabinoid receptors type 1 and 2 (CB1 and CB2) and activity of the serotonergic system [density of enterochromaffin cells (EC) and expression of the tryptophan hydroxylase isoform 1 and 2 (TPH1 and TPH2)] were characterized in the same animals. Finally, to determine if these alterations translate into functional changes in visceral sensitivity, we tested visceral pain-related responses in animals treated with antibiotics, with or without the addition of stress. For this, we assessed the presence of visceral pain-related behaviors associated with the intracolonic administration of capsaicin, as previously described.[9,10]

MATERIALS AND METHODS

Animals

Female C57BL/6N mice, 6 weeks old (Charles River Laboratories, Lyon, France) were used. Upon arrival, animals were acclimatized for a 1-week period prior to any experimentation. All animals were maintained in standard conditions in an environmentally controlled room (20–22 °C, 12 h light : dark cycle), with food and water *ad libitum*. 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 treatment

Animals received a mixture of non-absorbable, broad spectrum antibiotics containing Bacitracin A (Vetranal™; Sigma-Aldrich, Barcelona, Spain), and Neomycin (Neomycin trisulfate salt hydrate; Sigma-Aldrich). Amphotericin B (Sigma-Aldrich) was added to prevent yeast overgrowth. Animals were dosed by oral gavage with 0.3 mL of the antibiotic/antifungal mixture, during seven consecutive days. In addition, the same antibiotic/antifungal mixture was added to the drinking water during the same period of time. This protocol ensured a minimum dose of 0.4 mg for bacitracin and neomycin and 0.1 mg for amphotericin B (per mouse and day). Vehicle-treated animals received vehicle (deionized water) by oral gavage (0.3 mL) and normal drinking water during the same period of time. Water consumption, assessed on a daily basis during the treatment period, was similar across groups (data not shown). Similar treatment protocols have been followed previously in comparable studies in mice and rats, demonstrating the induction of significant changes of the commensal microbiota.[11–13]

Repetitive psychological stress (WAS)

Water avoidance stress was performed following previous protocols described by us.[14,15] Animals were placed on a platform (4 cm diameter, 6 cm height) located in the center of a standard plastic cage (530 × 280 × 155 mm) filled with tap water (18–20 °C) to about 1 cm below the edge of the platform. Stress sessions lasted for 1 h and were repeated on seven consecutive days. Control animals were maintained in their home cages. All procedures were performed in the morning (finishing no later than 12:00 h) to minimize any influence of circadian rhythms. Fecal pellet output during the 1-h session of WAS/non-stress was used as a marker of stress.

Behavioral responses to intracolonic capsaicin-evoked visceral pain

Spontaneous visceral pain-related behaviors induced by intracolonic capsaicin were assessed following previously described protocols, with minor modifications.[9,10] Mice were anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and capsaicin (0.05 mL per mice, 0.1% in ethanol : Tween 80 : saline; 1 : 1 : 8, v : v : v; Sigma-Aldrich) was administered intracolonically (about

 © 2013 John Wiley & Sons Ltd

4 cm from the anus) with a rounded tip plastic cannula (length 7.5 cm, diameter 0.61 mm). Petroleum jelly was applied on the perianal area to avoid stimulation of somatic areas through contact with capsaicin. Animals were placed in plastic cages (20 × 20 × 14 cm) and, after recovering consciousness, visceral pain-related behaviors were assessed during a 30-min period. Pain behaviors were visually assessed by two independent researchers. Behaviors assessed included: licking of the abdomen, stretching of abdomen, squashing the abdomen to the floor, and abdominal retractions. For each animal, the number of behaviors for the 30-min observation time was determined as the mean of the quantification performed by the two observers.

Experimental protocols

Mice ($n = 24$) were randomly divided into four experimental groups ($n = 6$ each): (i) vehicle-treated non-stressed mice; (ii) vehicle-treated stressed mice; (iii) antibiotic-treated non-stressed mice; and (iv) antibiotic-treated stressed mice. Animals were treated with antibiotics or vehicle for a period of 7 days, as described above. In addition, from day 2 to 8, animals were subjected to a 1 h per day session of psychological stress (WAS) or maintained in their home cages (control). On day 8, immediately after the last session of stress, animals were euthanized and blood, tissue (ceco-colonic region), and fecal samples were obtained.

In a second experiment, mice ($n = 20$) were divided into the same experimental groups and followed the same treatments ($n = 5$ per group). At the end of treatments, visceral pain-related responses to intracolonic capsaicin were assessed as described above. In this case, at the end of the procedure, animals were euthanized and weight of body organs was assessed (see samples collection).

Samples collection

Immediately after the last stress session, mice were deeply anesthetized with isoflurane (Isoflo; Esteve) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Thereafter, a medial laparotomy was performed, the ceco-colonic region localized and the cecum and colon dissected. Afterward, ceco-colonic fecal contents and a tissue sample from the proximal colon were collected and frozen immediately in liquid nitrogen. Frozen samples were stored at −80 °C until analysis. At the same time, tissue samples of the proximal and middle colon (about 1.5 cm each) were collected and fixed overnight in Carnoy fixative (ethanol : chloroform : glacial acetic acid, 6 : 3 : 1, v : v : v) or in 4% paraformaldehyde. After an overnight fixing, tissues were paraffin embedded and 5-μm-thick sections were obtained. In addition, the adrenal glands, the thymus, and the spleen were dissected and weighed. Serum was obtained by centrifugation of blood samples (15 min, 2465 g, 4°°C) and maintained at −80°°C until analysis. In animals used to assess visceral sensitivity, at necropsy, only the weight of body organs was assessed (cecum, adrenal glands, thymus, and spleen).

Bacterial identification by fluorescence *in situ* hybridization

For fluorescence *in situ* hybridization (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). Probes used were as follows: EUB 338 (5′GCTGCCTCCCGTAGG AGT3′) to total Bacteria; NON 338 (5′ACATCCTACGGGAGGC 3′) to non-bacteria (negative control); BAC 303 (5′CAATGTGGGG GACCTT3′) to *Bacteroides* spp.; EREC 482 (5′GCTTCTTAGT-CAGGTACCG3′) to *Clostridium* Cluster XIVa; LAB 158 (5′GG TATTAGCACCTGTTTCCA3′) to *Lactobacillus* spp. and *Enterococcus* spp.; ENT-D (5′TGCTCTCGCGAGGTCGCTTCTCTT3′) to enterobacteria; and BIF 164 (5′CATCCGGCATTACCACCC3′) to *Bifidobacterium* spp.

Fecal samples of ceco-colonic content were used to characterize luminal commensal microbiota. *In situ* hybridization of bacteria in the luminal content was performed on glass slides, as previously described.[16,17] Samples were hybridized in a dark moist chamber (for 3 h) by addition of 100 μL hybridization buffer (20 mmol L^{-1} Tris-HCl, 0.9 mol L^{-1} NaCl, 0.1% SDS at pH 7.2) with the corresponding Cy3-labeled oligonucleotide probe (concentration 5 ng μL^{-1}). Treatments with formamide or lysozyme and hybridization temperatures were used as described to achieve the optimal stringency. After hybridization, the slides were rinsed in a pre-warmed washing buffer (20 mmol L^{-1} Tris-HCl, 0.9 mol L^{-1} NaCl at pH 7.2) for 30 min and then cleaned with miliQ water to remove unbound probes. Washed slides were air dried and mounted with Vectashield-DAPI (Vector Laboratoires, Orton Southgate, Peterborough, UK). The fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI), that 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 (Carl Zeiss, Jena, Germany) 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[18], and the mean value obtained. All procedures were performed on coded slides to avoid bias.

Hybridization of tissue samples was performed following, with minor modifications, methods described by Pelissier *et al.*[19] Sections from Carnoy-fixed tissues were deparaffinized, rehydrated, post-fixed in 4% paraformaldehyde and washed. Hybridization conditions used were, essentially, as described above for luminal bacteria, but tissue samples were incubated for 16 h with the hybridization buffer. In hybridized tissue samples, 20 randomly selected fields were photographed. Analysis of the images was performed manually by two independent researchers who 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 15% of the pictures observed (at least three of 20) was required to decide that there was bacterial attachment to the epithelium. All procedures were performed on coded slides to avoid bias.

mRNA analysis

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit (Ambion/Applied biosystems, Foster City, CA, USA). Thereafter, a two-step quantitative real-time PCR (RT-qPCR) was performed. RNA samples were converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Only a consistent 260/280 ratio (between 1.8 and 2) found with NanoDrop (ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) was accepted to perform a TaqMan quantitative RT-qPCR. TaqMan gene expressions assays (hydrolysis probes) for CB1 receptors (Mm01212171_s1), CB2 receptors (Mm00438286_m1), interleukin

© 2013 John Wiley & Sons Ltd

6 (IL-6) (Mm00446190_m1), tumor necrosis factor α (TNFα; m00443258_m1), TPH1 (Mm00493 794_m1), and TPH2 (Mm00557715_m1) were used (Applied Biosystems). β-2-microglobulin (Mm00437762_m1) was used as endogenous reference gene.

The PCR reaction mixture was incubated on the ABI 7900 HT Sequence Detection System (Applied Biosystems). All samples, as well as the negative controls, were assayed in triplicates. RQ Manager 1.2 software was used to obtain the cycle threshold for each sample; thereafter, all data were analyzed with the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the vehicle–non-stressed group serving as the calibrator.[20]

Quantification of secretory immunoglobulin A

Luminal s-IgA was measured in fresh homogenates of cecal contents (diluted in PBS 1×) using a commercial double-antibody sandwich ELISA, following manufacturers' instructions (MBS 564073; MyBiosource, San Diego, CA, USA).

Histology

For histological examination, hematoxylin–eosin-stained sections from the colon were obtained following standard procedures. 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), and 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.

The mucous layer was assessed in Carnoy-fixed samples of colonic tissue. Thickness of the mucous layer was measured in 10 different fields, for triplicate, in representative regions covering, at least, 20% of the epithelial surface.[21] All measurements were performed on coded slides by two independent investigators using the Zeiss AxioVision Release 4.8.1 software. Moreover, tissue sections were also stained with Alcian Blue pH 2.5/Periodic Acid Schiff (AB 2.5/PAS kit; Bio-Optica, Milano, Italy) to specifically stain neutral (pink) and acidic (blue) mucins. Thereafter, colonic goblet cells were counted in 20 longitudinally oriented villus-crypt units. Length of the villus-crypt unit was also determined to obtain goblet cells density (number of cells mm^{-1}).

Immunohistochemistry

Immunohistochemistry was used to detect serotonin (5-HT) and Mouse Mast Cell Protease I (MMCP-I) in colonic tissue. The primary antibodies included a rabbit polyclonal anti-5-HT (1:20000; RA20080; Neuromics, Edina, MN, USA) and a sheep polyclonal anti-MMCP-I (1:500; MS-RM8; Moredun Scientific, Penicuik, Midlothian, Scotland). The secondary antibodies used were a biotinylated polyclonal swine anti-rabbit IgG (1:200; E 0353; DakoCytomation, Glostrup, Denmark) or a polyclonal rabbit anti-sheep IgG-B (1:200; SC-2776, Santa Cruz Biotechnology, Santa Cruz, CA, USA), as appropriate. Antigen retrieval for serotonin was achieved by microwave processing of the slides in 10 mmol L^{-1} citrate buffer. Quenching of endogenous peroxidase was performed by 1-h incubation with 5% H_2O_2 in distilled water. Detection was performed with avidin/peroxidase kit (Vectastatin ABC kit; Vector Laboratories). Antigen–antibody complexes were reveled with 3-3′-diaminobenzidine (SK-4100 DAB; Vector Laboratories). Specificity of the staining was confirmed by omission of the primary antibody.

For quantification, immunopositive cells were counted at high power field (hpf; 400× magnification) in 10 microscope fields, randomly selected, in duplicate, for each tissue sample. When assessing serotonin immunoreactivity, immunopositive cells, likely corresponding to EC, were counted in the mucosa. When assessing MMCP-I immunoreactivity, immunopositive cells, corresponding to mucosal mast cells, were counted in the mucosa and submucosa. All cell counting was performed on coded slides to avoid bias.

Plasma corticosterone and haptoglobin

Plasma corticosterone levels were determined by double-antibody RIA. The characteristics of the antibody and the basic RIA procedure had been described previously.[22] In brief, ^{125}I-corticosterone-carboximethyloxime-tyrosine-methyl ester (ICN-Biolink 2000, Barcelona, Spain), synthetic corticosterone (Sigma-Aldrich), as the standard, and an antibody raised in rabbits against corticosterone-carboximethyloxime-BSA were used. All samples were run in the same assay to avoid interassay variability. The intraassay coefficient of variation was less than 8% and the sensitivity was 0.1 μg dL^{-1}.

Plasma concentrations of the acute-phase protein haptoglobin were determined using a commercial ELISA kit, following manufacturer's instructions (sensitivity; 0.005 mg^{-1}; intraassay variability: 5.3–6.3%; interassay variability: 4.1–5.7%; "PHASE"™ Haptoglobin Assay; Tridelta Development Limited, Maynooth, County Kildare, Ireland).

Statistical analysis

Data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Data were analyzed by one-way ANOVA or a non-parametric ANOVA (visceral pain data), followed, when necessary, by a Student–Neuwman–Keuls multiple comparisons test. Data were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Functional and endocrine responses to repetitive psychological stress (WAS)

In vehicle-treated mice maintained in non-stressful conditions, pellet output was low and not affected by the antibiotic treatment (mean value for the 7 days of stress; vehicle: 3.4 ± 0.6 fecal pellets h^{-1}; antibiotic: 3.9 ± 0.7 fecal pellets h^{-1}; $P > 0.05$; Fig. 1A). Repetitive WAS, independently of the experimental group

 © 2013 John Wiley & Sons Ltd

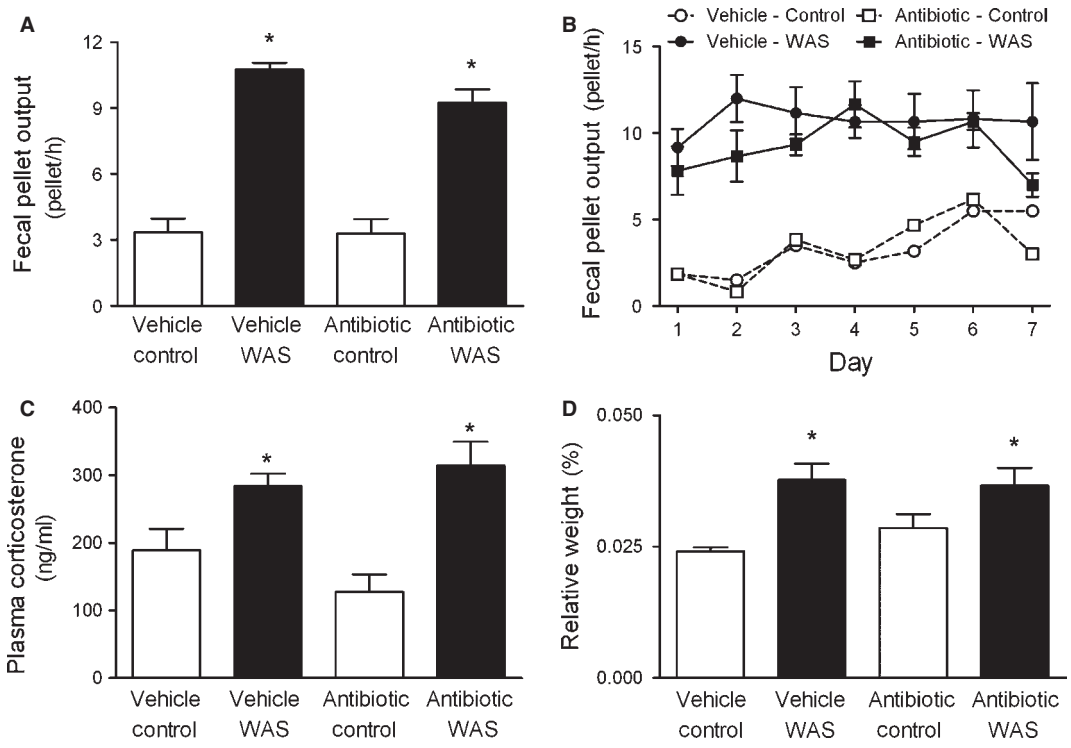


Figure 1 Functional and endocrine responses to repetitive water avoidance stress (WAS, 1 h day^{-1} for 7 days) in mice. (A) Mean fecal pellet output during the time of stress, across the 7 days of treatment. $^{*}P < 0.05$ *vs* non-stressed groups. (B) Mean fecal pellet output for the seven WAS sessions applied. (C) Plasma levels of corticosterone at the end of the last stress session. $^{*}P < 0.05$ *vs* non-stressed groups. (D) Weight of the adrenal glands. $^{*}P < 0.05$ *vs* vehicle–non-stressed group. In all cases data are mean ± SEM, $n = 6$ per group (except for the weight of the adrenal glands, $n = 11$ per group).

considered, resulted in a significant increase in the fecal output rate during the period of stress, when compared with non-stressed groups (Fig. 1A). Defecatory response to stress was similar in vehicle- and antibiotic-treated animals and remained stable during the seven consecutive stress sessions (Fig. 1B).

Plasma corticosterone levels were increased in stressed animals, as assessed immediately after the last stress session. Stress-induced changes in plasma corticosterone were similar in vehicle- or antibiotic-treated mice (Fig. 1C).

Weight of body organs

At necropsy, weight of the adrenal glands was increased by 50% in the vehicle-WAS group when compared with non-stressed controls (Fig. 1D). Antibiotic treatment, *per se*, resulted in a slight increase in the adrenal glands weight, without reaching statistical significance. Addition of stress in antibiotic-treated mice leads to an increase in adrenal weight similar to that observed in vehicle-treated animals (Fig. 1D). The same differences were observed for the relative weight of the adrenal glands. No consistent changes across groups were observed in the absolute or relative weight of the spleen or the thymus (Fig. 2C).

Macroscopic and microscopic evaluation of ceco-colonic tissues

In antibiotic-treated groups, irrespective of the addition of stress, the cecum appeared distended and its weight was significantly increased when compared to that of the vehicle–non-stressed group (Fig. 2A). These differences persisted when the relative weight of the cecum was calculated (data not shown), thus indicating that the increase in cecal weight was independent of a variation in bodyweight.

Upon macroscopical examination, both the cecum and colon showed a normal aspect, irrespective of the experimental group considered. Colonic relative weight was similar across groups (Fig. 2A). Overall, microscopic analysis of colonic tissue samples showed a normal histological structure, irrespective of the experimental group considered. Occasionally, a moderate multifocal-to-diffuse inflammatory infiltrate

© 2013 John Wiley & Sons Ltd

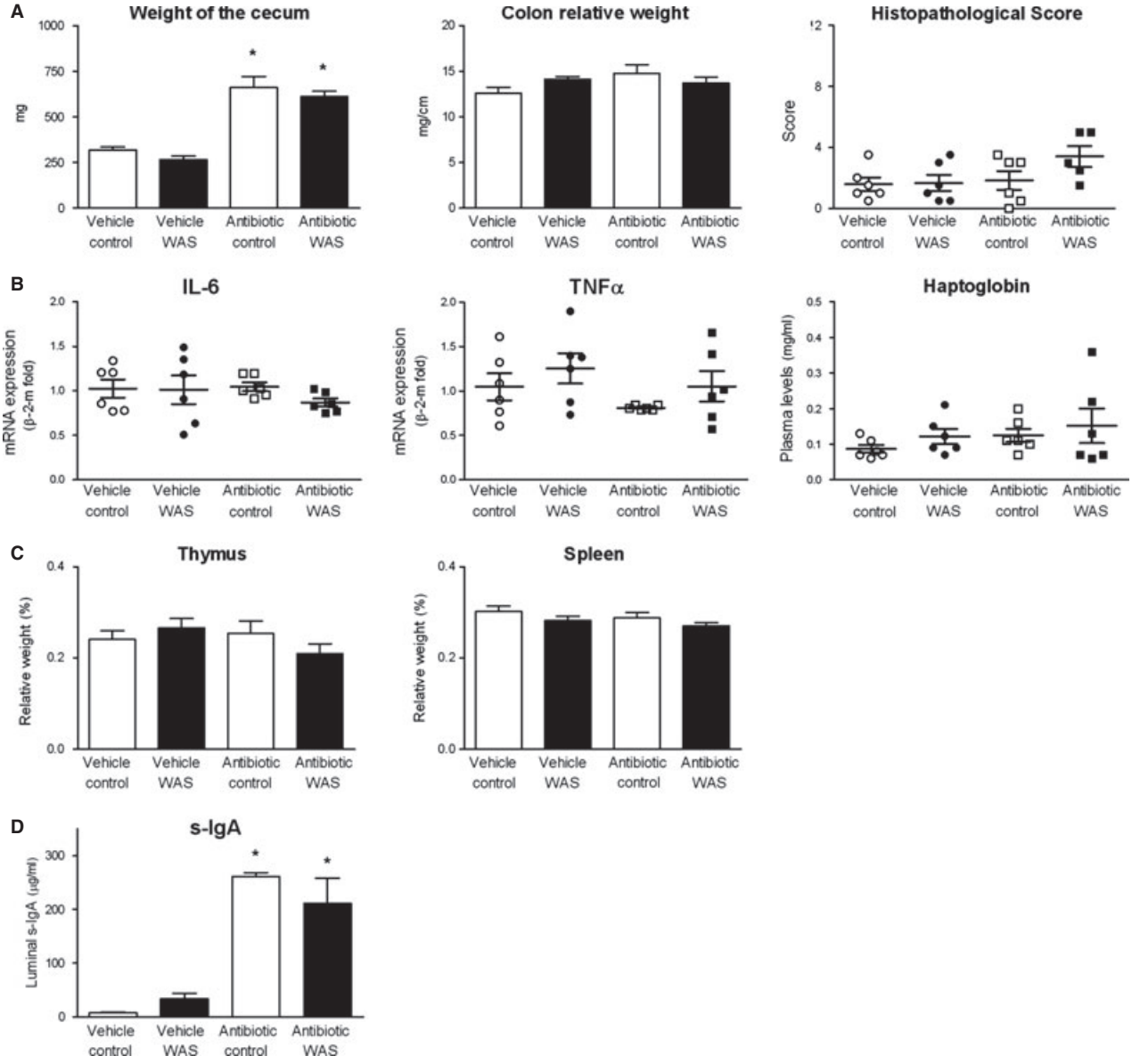


Figure 2 Ceco-colonic histopathology and immune-related parameters at the time of necropsy in the different experimental groups. (A) (top row): Histopathological evaluation: weight of the cecum (left panel); relative weight of the colon (middle panel), and colonic histopathological scores (right panel). Data are mean ± SEM of 5–11 animals per group. Because of technical problems the weight of the cecum in the vehicle–WAS group was only assessed for five animals; and histopathological scores were not determined in one animal of the antibiotic-treated–WAS group. $^{*}P < 0.05$ *vs* vehicle–non-stressed group. (B) (second row): Local and systemic inflammatory markers: colonic expression of IL-6 (left panel) and TNFα (middle panel) and plasma levels of haptoglobin (right panel). Each point represents an individual animal; the horizontal bar with errors represents de mean ± SEM; $n = 6$ per group. (C) (third row): Relative weight (% of total bodyweight) of the thymus (left panel) and the spleen (right panel). Data are mean ± SEM of 11 animals per group. (D) (bottom row): Luminal secretory-IgA (s-IgA) in the different experimental groups. Data are mean ± SEM of six animals per group. $^{*}P < 0.05$ *vs* vehicle-treated groups.

could be observed, but no treatment-related incidence could be established. Final histopathological scores were similar in all experimental groups (Fig. 2A). Nevertheless, total histopathological scores assigned to the antibiotic–WAS group were relatively high compared with other groups; however, no statistical significance was reached [$F(3.19) = 2.090$; $P = 0.135$]. Increased scores in this group were mainly associated with a worsening in the epithelial structure with increased desquamation and scant alterations in some of the crypts [$F(3.19) = 3.116$; $P = 0.048$ antibiotic-treated–non-stressed *vs* antibiotic-treated–WAS]. No differences among groups were found in the length of the colonic crypts.

© 2013 John Wiley & Sons Ltd

Table 1 Effect of stress and/or antibiotics on the colonic mucous layer and the density of goblet cells

	Thickness of the mucous layer (μm)	Goblet cells density (cells mm^{-1})	Density of Mature goblet cells (cells mm^{-1})
Vehicle–Non -stressed	23.01 ± 1.89	99.19 ± 7.45	20.11 ± 1.90
Vehicle–WAS	11.87 ± 0.32*	114.06 ± 8.99	29.88 ± 0.85*
Antibiotic –Non -stressed	17.99 ± 0.72	113.75 ± 2.32	24.48 ± 1.32
Antibiotic –WAS	7.34 ± 0.27*,†	111.25 ± 9.45	25.04 ± 4.6

Data are mean ± SEM, n = 6 animals per group; WAS, water avoidance stress.
*$P < 0.05$ *vs* vehicle–non-stressed.
†$P < 0.05$ *vs* antibiotics–non-stressed.

Very few MMCP-1-immunopositive cells (0–1 cells per field), identified as mucosal mast cells, were observed in colonic samples, irrespective of the experimental group considered (data not shown).

In Carnoy-fixed colonic samples, a layer of mucous was observed covering most of the epithelial surface. Stress decreased in a similar proportion the thickness of the mucous layer in either vehicle- or antibiotic-treated mice. Antibiotics, *per se*, had only a marginal, non-significant, effect reducing the thickness of the mucous layer (Table 1). Despite these changes, the density of goblet cells was similar across experimental groups. The relative abundance of mature goblet cells containing neutral mucins (pink color in a PAS/AB pH = 2.5 staining) was slightly increased by either antibiotic treatment or stress, although statistical significance was only achieved for the stress group (Table 1).

Systemic and local markers of inflammation and luminal s-IgA

Plasma levels of the acute-phase protein haptoglobin were, in general, low and similar to those previously described by us.[15] No treatment-related changes in haptoglobin levels were found among groups (Fig. 2B). Similarly, no differences among groups were found for colonic cytokines mRNA expression (IL-6 and TNFα; Fig. 2B). In most cases, there was relatively large within-group variability in the expression levels. Overall, relative expression of TNFα was higher (by 12-fold) than that of IL-6.

S-IgA was detected in all fecal samples, regardless the experimental group considered. In vehicle–non-stressed animals, s-IgA levels were 7.19 ± 1.3 μg mL^{-1}, the addition of stress increased s-IgA levels by 4.6-fold (Fig. 2D), although statistical significance was not reached. In the antibiotic–non-stressed group, s-IgA levels were increased by 36-fold ($P < 0.05$ *vs* vehicle–non-stress group). In these conditions, addition of stress did not further enhance the levels of s-IgA (Fig. 2D).

Characterization of luminal and wall-adhered microbiota

In vehicle-treated–non-stressed animals, total bacterial counts within the luminal content, determined by FISH as EUB338-positive cells, were between 3×10^{10} and 7×10^{10} cell mL^{-1}, and within the margins previously described.[21,23,24] In these conditions, EUB338-positive bacteria represented a 90% of the total DAPI counts (Table 2). Within all bacterial groups characterized, *Bacteroides* spp. and *Clostridium* spp. were the

Table 2 Composition of the luminal microbiota as assessed by FISH and DAPI staining§

	DAPI ($\times 10^8$ cells mL^{-1})	Total bacteria ($\times 10^8$ cells mL^{-1})	*Bacteroides* spp. ($\times 10^8$ cells mL^{-1})	Enterobacteria ($\times 10^8$ cells mL^{-1})	Verrucobacteria ($\times 10^8$ cells mL^{-1})	*Clostridium coccoides* cluster XIVa ($\times 10^8$ cells mL^{-1})	*Lactobacillus-Enterococcus* spp. ($\times 10^8$ cells mL^{-1})	*Bifidobacterium* spp ($\times 10^8$ cells mL^{-1})
Vehicle –Non -stressed	462.5 ± 56.0	433.5 ± 27.6	69.3 ± 3.7	ND	36.5 ± 2.3	68.7 ± 4.9	ND	ND
Vehicle–WAS	532.2 ± 55.7	509.0 ± 26.8	64.3 ± 5.9	ND	ND	110.0 ± 8.4‡	0.1 ± 0.02	ND
Antibiotic –Non -stressed	286.9 ± 22.9	173.5 ± 10.5*	73.7 ± 8.4	7.0 ± 0.6*	18.4 ± 1.2*	31.4 ± 1.6*,†	18.6 ± 1.7*	ND
Antibiotic –WAS	341.3 ± 22.0	196.8 ± 8.1*	103.0 ± 9.5	11.0 ± 0.9*,†	44.4 ± 1.7†	18.8 ± 1.2*,†	47.2 ± 3.8*,†	ND

ND, Not detected (below 10^6 cells mL^{-1}); FISH, fluorescent *in situ* hybridization; DAPI, 4′,6-diamidino-2-phenylindole; WAS, water avoidance stress.
*$P < 0.05$ *vs* vehicle–non-stressed or vehicle–WAS groups.
†$P < 0.05$ *vs* antibiotic–non-stressed group.
‡$P < 0.05$ *vs* vehicle–non-stressed group.
§Data represent mean ± SEM from six animals per group.

© 2013 John Wiley & Sons Ltd

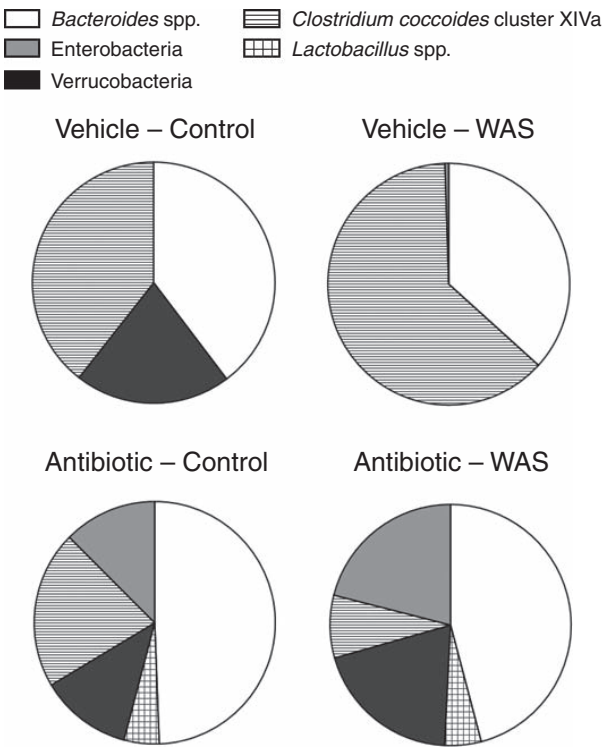


Figure 3 Relative distribution of the ceco-colonic microbiota in the different experimental groups. Data represent the relative abundance (percent) of the main bacterial groups present in the gut commensal microbiota (*Bacteroides* spp., *Clostridium* spp., Enterobacteria, *Lactobacillus* spp., and Verrucobacteria), as quantified using fluorescent *in situ* hybridization (FISH) techniques. Relative percent composition of the microbiota was calculated taking as 100% the total counts of the different bacterial groups assessed. See Table 2 for exact cell counts.

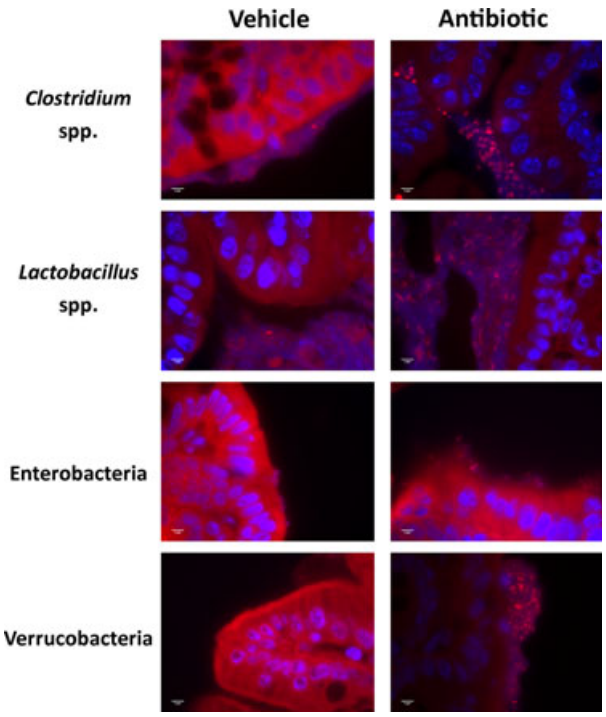


Figure 4 Representative colonic tissue images showing bacterial wall adherence for different bacterial groups. The left column corresponds to a vehicle-treated mice and the right column to an antibiotic-treated animal. Each line corresponds to a different bacterial group (from top to bottom: *Clostridium* spp, *Lactobacillus* spp, Enterobacteria, and Verrucobacteria). Note the higher abundance of bacteria attached to the epithelium and within the mucous layer covering the epithelial surface in the antibiotic-treated mice compared with the vehicle-treated mice.

most abundant strains; whereas Enterobacteria, *Lactobacillus/Enterococcus* spp. and *Bifidobacterium* spp. were below FISH detection levels (10^6 cell mL^{-1})[25] (Table 2; Fig. 3). Repetitive WAS had no effect, *per se,* on total bacterial counts, but induced a specific dysbiosis of the microbiota. In particular, Verrucobacteria counts were reduced to undetectable levels whereas counts of *Clostridium* spp. were increased by twofold and *Lactobacillus/Enterococcus* spp. appeared at a low level, borderline to the limit of detection (Table 2; Fig. 3).

Treatment with antibiotics resulted in a 2.5-fold reduction in total bacterial counts and altered the overall composition of the luminal microbiota (Table 2). In antibiotic-treated mice, EUB338-positive counts only included a 60% of the total DAPI counts. Antibiotics reduced the counts of Verrucobacteria and *Clostridium* spp., while significantly increased the counts of Enterobacteria and *Lactobacillus/Enterococcus* spp. (Table 2; Fig. 3). Addition of stress to the antibiotic treatment further enhanced intestinal dysbiosis. In these conditions, total bacterial counts maintained their reduction when compared with the

Table 3 Incidence of bacterial wall adherence*

	Bacteroides spp.	Enterobacteria	Verrucobacteria	*Clostridium coccoides* cluster XIVa	*Lactobacillus-Enterococcus* spp
Vehicle–Non-stressed	0/6 (0%)	0/6 (0%)	5/6 (83%)	1/6 (17%)	0/6 (0%)
Vehicle–WAS	0/6 (0%)	0/6 (0%)	1/6 (17%)	2/6 (33%)	0/6 (0%)
Antibiotic–Non-stressed	0/6 (0%)	6/6 (100%)	5/6 (83%)	5/6 (83%)	5/6 (83%)
Antibiotic–WAS	3/6 (50%)	2/6 (33%)	5/6 (83%)	6/6 (100%)	5/6 (83%)

*Data represent the number of animals showing bacterial wall adherence over the total of animals (percentage of incidence).

© 2013 John Wiley & Sons Ltd

vehicle–WAS group. This was associated mainly with a sixfold reduction in *Clostridium* spp. counts, whereas the counts of Verrucobacteria, Enterobacteria and *Lactobacillus/Enterococcus* spp. were significantly increased (Table 2; Fig. 3). *Bifidobacterium* spp. was not detected in any experimental group.

As it relates to bacterial wall adherence, EUB338-positive cells were always observed attached to the wall, in most cases within the mucous layer located on the epithelial surface. In vehicle–non-stressed animals, the only bacterial group attached to the colonic wall was Verrucobacteria (Incidence: 83%). Addition of stress significantly reduced the incidence of Verrucobacteria attachment (Table 3, Fig. 4), without affecting the adherence of other bacterial groups.

During antibiotic treatment, the incidence of bacterial wall adherence increased significantly for all bacterial groups detected in the luminal content (83–100% incidence), except for *Bacteroides* spp. (0% incidence). The addition of stress maintained a generalized adherence for all groups explored, but, particularly, facilitated *Bacteroides* spp. attachment while reduced the adherence of Enterobacteria (Table 3, Fig. 4).

Expression of Cannabinoid receptors and activity of the serotonergic system

mRNA for both cannabinoid receptors was detected in all samples. Expression levels in control conditions (vehicle–non-stressed animals) were low, with the levels of CB1 mRNA being about 10-fold higher than those of CB2. In vehicle-treated mice, WAS had a marginal effect increasing CB2 expression (by 6%, $P > 0.05$). In the antibiotic–non-stress group, CB2 expression was increased by 20% ($P < 0.05$ *vs* vehicle–non-stress group); addition of stress further increased CB2 expression, leading, approximately, to a 40% increase in expression ($P < 0.05$ *vs* vehicle-treated groups; Fig 5A). CB1 expression was not affected by either stress or antibiotics, alone or in combination (Fig 5A). Regardless of the experimental group considered, expression levels of CB2 receptors correlated positively with *Lactobacillus* spp. counts ($P = 0.001$; $r^2 = 0.38$) and negatively with *Clostridium* spp. counts ($P = 0.02$; $r^2 = 0.21$; Fig. 5B).

The isoform 1 of the TPH was detected with high reproducibility and at relatively high levels in colonic tissues (Fig. 5C); however, the isoform 2 (TPH2) was found in a very low quantity (a mean of 36.6 Cq value). Overall, TPH1 expression levels were about 24-fold higher than those of TPH2. TPH1 expression levels were similar in vehicle-treated or antibiotic-treated non-stressed animals. Repetitive WAS increased TPH1 expression by similar proportion in either vehicle- or antibiotic-treated animals (40% increase; Fig. 5C).

Serotonin-immunopositive cells, likely corresponding to EC cells, were scattered throughout the colonic mucosa. Relative abundance was similar in all experimental groups (Fig. 5C).

Behavioral responses to intracolonic capsaicin

Intracolonic administration of capsaicin induced pain-related behaviors in all mice during the 30-min observation period. The behavior most expressed was the licking of the abdominal area, which was observed in all animals. In the vehicle–non-stress group, the number of pain-related behaviors reached a mean value of 40.9 ± 6.6 in the 30-min observation period ($n = 5$; Fig. 5D). In these conditions, treatment with antibiotics slightly reduced the number of pain behaviors, although statistical significance was not reached. In vehicle-treated animals, addition of stress increased the incidence of behaviors by 48% ($P < 0.05$ *vs* vehicle–non-stress group), an effect completely prevented by the treatment with antibiotics (Fig. 5D).

DISCUSSION

In this study, we show that the colonic functional (motor) and endocrine responses to stress are essentially not affected by relatively large alterations of the ceco-colonic microbiota, either luminal or attached to the colonic wall, during an antibiotic treatment. Moreover, we show that microbiological changes, due to antibiotics and stress, are able to modulate the immune and sensory systems, namely the endocannabinoid and the serotonergic systems, within the colon, without the induction of a manifest state of intestinal inflammation. While antibiotics, *per se*, did not affect visceral pain-related responses, they prevented stress-induced hypersensitivity. This suggests that antibiotics-mediated effects on sensory systems might have functional consequences, leading to the modulation of visceral sensitivity.

Our results confirm the validity of chronic WAS as a valid, mild stressor in mice, as previously published.[14,15,26] Mice did not habituate to the stress protocol, as shown by the persistent colonic response along the 7-day period of WAS. Moreover, the efficacy of the stress paradigm is further demonstrated by the raise in plasma corticosterone and the increase in weight of the adrenal glands at the end of the last stress session.

Total bacterial counts were not affected by stress. However, repetitive WAS significantly increased the

© 2013 John Wiley & Sons Ltd

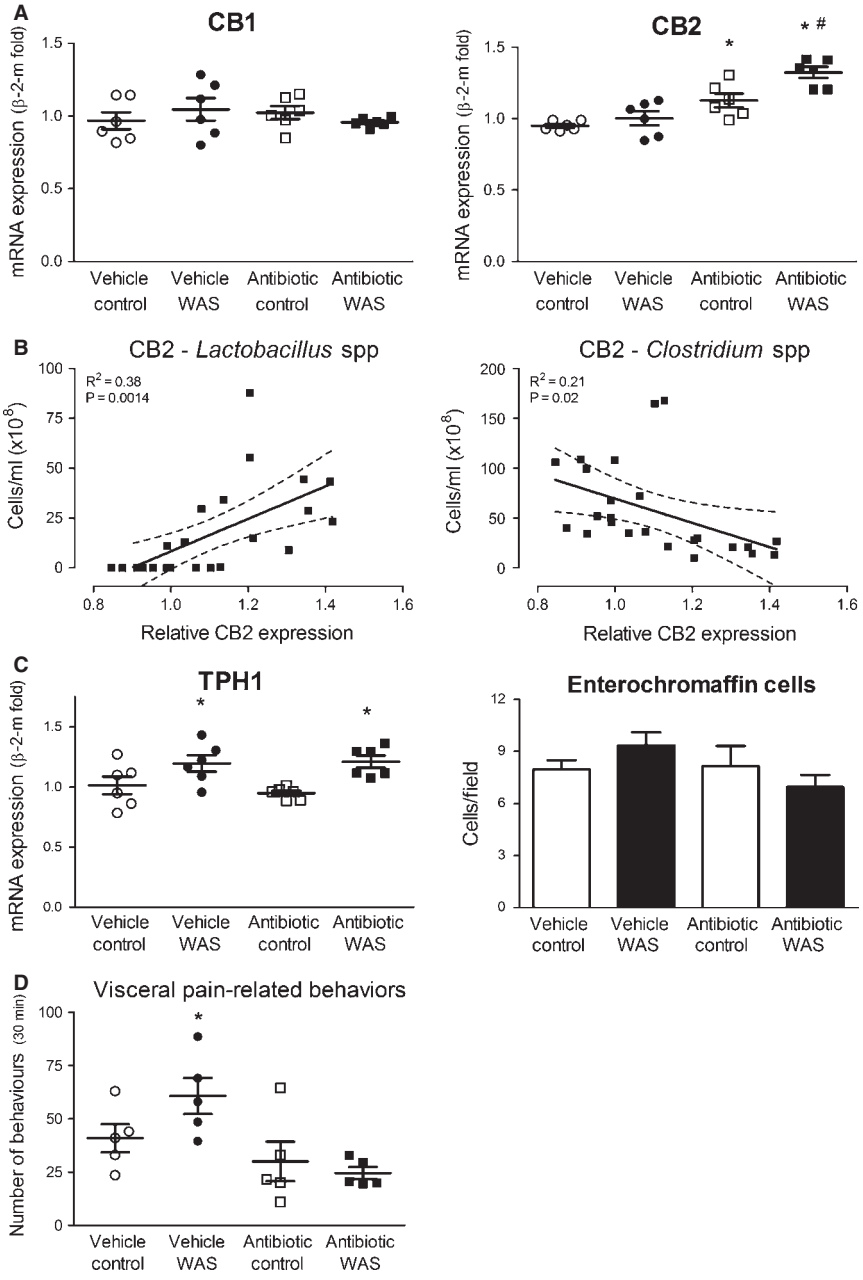


Figure 5 Effects of stress and/or antibiotics on the colonic expression of sensory-related systems (endocannabinoid and serotonergic) and visceral pain-related responses. (A) (upper row): Colonic expression of cannabinoid receptors, CB1 (left pane) and CB2 (right panel), in the different experimental groups. Each point represents an individual animal; the horizontal bar with errors represents de mean ± SEM of each group; $n = 6$ per group. $^{*}P < 0.05$ *vs* vehicle–non-stressed and vehicle–WAS groups. $^{\#}P < 0.05$ *vs* antibiotic–non-stressed group. (B) (middle-upper row): Correlations between the relative expression of CB2 and the bacterial counts of *Lactobacillus* spp. ($P = 0.0014$; $r^2 = 0.38$) (left panel) and *Clostridium* spp. ($P = 0.02$; $r^2 = 0.21$) (right panel), as determined by fluorescent *in situ* hybridization. Each point represents an individual animal; broken lines represent the 95% confidence interval. (C) (middle-lower row): Activity of the serotonergic system within the colon. Left panel shows the relative expression of tryptophan hydroxylase 1 (TPH1). Each point represents an individual animal; the horizontal bar with errors represents the mean ± SEM of each group; $n = 6$ per group. $^{*}P < 0.05$ *vs* respective non-stressed group. Right panel shows the density of enterochromaffin cells (5-HT-immunoreactive cells/field, ×400), as determined by immunohistochemistry, in the different experimental groups. Data are mean ± SEM of $n = 6$ animals per group. (D) (lower row): Intracolonic capsaicin-evoked visceral pain-related behaviors. Data represent the number of pain-related behaviors in a 30-min observation period after intracolonic capsaicin administration. Each point represents an individual animal; the horizontal bar with errors represents the mean ± SEM of each group; $n = 5$ per group. $^{*}P < 0.05$ *vs* other experimental groups.

© 2013 John Wiley & Sons Ltd

counts of *Clostridium* spp. and favored the appearance of *Lactobacillus* spp. These changes agree with those described in mice subjected to social stress, where the main change in the microbiota was an increase in the Clostridia group.[13] Interestingly, the Verrucobacteria group, present in a relatively high proportion in non-stressed mice, was undetectable in stressed animals. This group of microorganisms, which degrade mucus within the gastrointestinal tract,[17,27] might have relevance in gastrointestinal diseases. For instance, an enhancement of the mucin-degrading microbiota in dysbiotic patients predispose to Crohn's disease.[28] During stress, the thickness of the mucus layer was reduced, in agreement with O'Malley *et al.*[28,29] A reduction in mucus abundance might be a factor reducing also the relative abundance of Verrucobacteria. Alternatively, we cannot discard that these changes are secondary to the combined enhancing effects of stress on colonic motility and mucus secretion,[30–32] leading to an increased discharge of mucus and therefore to a net reduction in mucus content and associated bacteria. Moreover, although goblet cell density remained stable, stress increased the proportion of mature goblet cells, indicative of an increase in mucus production and secretion.[33] Despite these changes in mucus content, wall-adhered microbiota was not affected by stress.

As expected, treatment with wide-spectrum, non-absorbable antibiotics significantly reduced total bacterial counts. The reduction in bacterial counts was coupled to a specific dysbiosis which implied a proliferation of *Lactobacillus* spp. and Enterobacteria; whereas the *Clostridium* spp. and the Verrucobacteria groups were reduced. Interestingly, only antibiotic-induced changes in luminal microbiota were associated with an increase in bacterial wall adherence. This is important because adhered microbiota has been suggested to be the one directly interacting with the host's bacterial recognition systems, thus eliciting either beneficial or harmful responses within the gut.[34,35] The relationship between luminal counts and epithelial attachment seems to be strain dependent. Overall, changes in bacterial wall adherence correlated positively with changes in luminal counts. However, the Clostridia group was reduced during antibiotic treatment, but presented an increased rate of adherence. This negative relationship might reflect the heterogeneity of *Clostridium coccoides* cluster XIVa. From the present data, we cannot rule out the possibility that antibiotics are affecting only a part of this cluster, leading to a relative selection of bacteria with high wall adherence capacities. In fact, it is well reported that most antibiotics can increase the risk of developing *Clostridium difficile* colitis[36,37] and that the relapse of colitis in patients with recurrent *C. difficile* infections is associated with reduced intestinal microbial diversity.[38] Nevertheless, the role of gut commensal microbiota in intestinal inflammation remains controversial, and beneficial effects of wide spectrum antibiotics has been shown in DSS-induced colitis in rats.[39] The mucous layer represents also a protective barrier preventing bacterial wall adherence. Therefore, a loss of mucus should be regarded as a factor favoring bacterial–host interactions.[40,41] Antibiotics had only a marginal effect reducing the mucous layer, thus suggesting that the mucus, *per se*, might play a minor role affecting bacterial wall adherence in the present conditions. Ceco-colonic dysbiosis was further enhanced when antibiotic-treated mice were subjected to stress. This was associated with a significant increase in the incidence of wall adherence, observed for all bacterial groups assessed, and a clear reduction in the thickness of the mucous layer.

Commensal microbiota is necessary for the development of spontaneous colitis, as suggested by observations in mice deficient in interleukin 10; however, gut commensal microbiota could also have a protective role, as seen in germfree mice with DSS-induced colitis.[42–44] These apparent discrepancies might be associated with the composition of the microbiota, the immaturity of the immune system, the environmental conditions of housing, and the type of treatment applied (duration and antibiotics used). In any case, the potential pathophysiological implications of these observations warrant further investigations. In humans, increased bacterial wall adherence has been suggested as a pathogenic factor leading to local immune responses that favor the appearance and maintenance of intestinal inflammation.[21,45] Interestingly, antibiotic-induced dysbiosis had no impact on the gut-to-brain modulation of endocrine responses to psychological stress. This agrees with recent data suggesting that the gut-to-brain signaling is established during the early post-natal phase and that commensal microbiota is important during that imprinting period.[4,46] Once the gut is colonized and the commensal microbiota established, changes in microbiota composition seem to have a minor impact in gut-to-brain signaling, at least as stress-related endocrine responses relates.[46] Despite this, intestinal microbiota has been related as a putative factor affecting gut sensory systems leading to altered behavioral[47,48] and local visceral responses, such as visceral pain.[12,49] For instance, gut commensal microbiota is fundamental for the development of inflammatory pain in

© 2013 John Wiley & Sons Ltd

mice.[12,49,50] Here, we assessed changes in the endocannabinoid and the serotonergic systems, two of the main sensory systems within the gut, with a demonstrated involvement in secretomotor- and visceral pain-related responses.[49,51–54] In the present conditions, antibiotics selectively upregulated the expression of CB2; an effect further enhanced by the addition of stress. This agrees with data suggesting that gut microbiota is able to upregulate the endocannabinoid system within the gut.[55] Modification in the commensal microbiota by addition of specific bacterial strains (namely *L. acidophilus*) has been shown to upregulate CB2 expression in rats and mice, leading to the induction of visceral analgesia.[49] In agreement with this, changes in CB2 expression correlated positively with luminal counts of *Lactobacillus* spp., which increased with antibiotic treatment and were further enhanced in stressed antibiotic-treated mice. Overall, these observations further support the view that bacteria of the *Lactobacillus* spp. group should be regarded as a beneficial component of the microbiota, which might be implicated in the modulation of visceral pain responses through the modulation of the intestinal endocannabinoid system. On the other hand, counts of *Clostridium* spp. correlated in a negative manner with the CB2 expression reinforcing the potential role assigned to this bacterial group as a pathogenic component of the microbiota.

Expression of TPH1 and TPH2 and density of EC cells served to assess the activity of the serotonergic system. As expected, expression of TPH2, the isoform responsible for the synthesis of neuronal serotonin, was very low in whole colonic homogenates. On the other hand, expression TPH1, responsible for serotonin synthesis in EC cells, was detected at relatively high levels. Interestingly, TPH1 was upregulated in stressed animals, independently of the antibiotic treatment. These observations might suggest that, although not directly assessed, serotonin synthesis and availability is increased during stress, with commensal microbiota playing a minor role *per se*. Overall, this agrees with studies showing that serotonin availability might be increased within the colon during stress.[56] However, density of EC cells was not affected by stress, thus suggesting a cellular hyperactivity, rather than a hyperplasia. This contrasts with inflammatory models of gut dysfunction, such as the experimental infection with *Trichinella spiralis*, in which increased availability of serotonin has been associated with a hyperplasia of EC cells.[57,58] The functional consequences of these changes in the cannabinoid and serotonergic systems warrant further studies, outside the original scope of the present work.

The changes observed in the expression of sensory-related systems are likely to have a functional significance. This is demonstrated by the changes in visceral pain-related responses observed in antibiotic-treated *vs* non-treated animals. In agreement with previous reports, we show that intracolonic capsaicin evokes behavioral responses consistent with the induction of visceral pain.[9,10] Moreover, an increase in pain-related events was observed in stressed animals, thus confirming data indicating that repeated psychological stress induces visceral hypersensitivity in rodents.[26,59] Interestingly, stress-induced hyperalgesic responses were completely prevented by the treatment with antibiotics. However, in non-stressed animals, antibiotics had no significant effects on visceral pain-related behaviors. This might suggest that the modulatory effects exerted by antibiotics are able to compensate states of altered (increased) sensitivity, without affecting basal responses. Therefore, it is feasible to assume that the changes observed in CB2 expression and serotonin availability might lead to functional effects modulating states of altered visceral sensitivity. Similarly, other sensory mediators not directly assessed here and involved in visceral pain responses, such as vanilloids,[60] might be involved in the responses observed. Overall, these observations further support an involvement of gut microbiota as a modulatory component of gut sensory functions.

As mentioned, none of the treatments applied resulted in evident intestinal inflammation. Although enlargement of the cecum was observed in antibiotic-treated animals, this was not associated with consistent histopathological alterations. It is interesting to point out that despite the increased host–bacterial interaction observed in dysbiotic mice, no signs of colonic inflammation (either macroscopical, microscopical or biochemical) were observed following the treatment with antibiotics. This contrasts with previous reports that observed signs of intestinal inflammation during both antibiotic treatment and stress.[12,41,61,62] In particular, the appearance of stress-induced intestinal inflammation has been related with a mast cell infiltrate and the facilitation of bacterial wall adherence in rats.[12,41,61,62] However, in our conditions, the density of mast cells was not increased by stress. Although inflammatory markers were unaltered, luminal s-IgA levels were increased during dysbiosis. Luminal s-IgA contributes to the suppression of immune reactions generated by commensal bacteria[63,64] and, when binding to bacteria, prevent bacterial translocation.[65] Increased s-IgA levels might represent a mucosal response, likely triggered by the increased rate of bacterial attachment during dysbiosis,

 © 2013 John Wiley & Sons Ltd

aiming the prevention of local and systemic inflammation and bacterial translocation. Multiple factors ranging from the species/strain used to the intensity of the stressors applied or the microbial environment might contribute to the final immune response to a dysbiotic state. Systematic studies addressing these aspects will be necessary to determine the relative contribution of these factors to the final responses observed within the gut.

In summary, the current study shows that gut commensal microbiota and stress are likely to act as interactive components in the maintenance of gut homeostasis and in the development of gut pathophysiology. Changes observed here suggest that microbiota and stress are able to selectively modulate gut sensory mechanisms, in the absence of obvious structural or biochemical alterations compatible with the presence of intestinal inflammation. Nevertheless, a mucosal immune response, characterized by increased s-IgA production, could be observed. Moreover, the treatment with antibiotics was associated with a reduction in stress-induced visceral hypersensitivity, thus suggesting that microbiota, influencing sensory-related systems within the gut, is able to modulate visceral pain arising from the intestine. Overall, these data support the potential involvement of stress and gut microbiota in the alterations observed in patients with functional gastrointestinal disorders, characterized by secretomotor and sensory alterations in the absence of structural changes. These observations warrant further studies dissecting the pathways altered by stress and gut microbes and the associated functional changes. Our observations support the view that the beneficial effect of certain bacterial strains, used as probiotics, might be associated with the modulation of the activity of endogenous sensory-related systems, such as the endocannabinoid system.

ACKNOWLEDGMENTS

E. Martínez and A. Acosta are thanked for their technical assistance.

FUNDING

This study was supported by grants BFU2009-08229 and BES-2010-037699 (FPI program; M. A. personal support) from the Ministerio de Ciencia e Innovación (Spain) and 2009SGR708 from the Generalitat de Catalunya.

DISCLOSURE

The authors do not have any conflict of interest.

AUTHOR CONTRIBUTION

MA and VM designed and performed experiments, analyzed data, and wrote the manuscript; PV participated in the discussion of the data; All authors approved the final version of the manuscript.

REFERENCES

1 Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480–91.

2 Elsenbruch S. Abdominal pain in Irritable Bowel Syndrome: a review of putative psychological, neural and neuro-immune mechanisms. *Brain Behav Immun* 2011; **25**: 386–94.

3 Barbara G, Cremon C, De Giorgio R *et al.* Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. *Curr Gastroenterol Rep* 2011; **13**: 308–15.

4 O'Mahony SM, Marchesi JR, Scully P *et al.* Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol Psychiatry* 2009; **65**: 263–7.

5 Bercik P, Collins SM, Verdu EF. Microbes and the gut-brain axis. *Neurogastroenterol Motil* 2012; **24**: 405–13.

6 DuPont AW, DuPont HL. The intestinal microbiota and chronic disorders of the gut. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 523–31.

7 Mantis NJ, Rol N, Corthesy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol* 2011; **4**: 603–11.

8 Hansen J, Gulati A, Sartor RB. The role of mucosal immunity and host genetics in defining intestinal commensal bacteria. *Curr Opin Gastroenterol* 2010; **26**: 564–71.

9 Gonzalez-Cano R, Merlos M, Baeyens JM, Cendan CM. Sigma1 receptors are involved in the visceral pain induced by intracolonic administration of capsaicin in mice. *Anesthesiology* 2013; **118**: 691–700.

10 Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F. A new model of visceral pain and referred hyperalgesia in the mouse. *Pain* 2001; **92**: 335–42.

11 van der Waaij D, Berghuis-de Vries JM, Korthals Altes C. Oral dose and faecal concentration of antibiotics during antibiotic decontamination in mice and in a patient. *J Hyg* 1974; **73**: 197–203.

12 Verdu EF, Bercik P, Verma-Gandhu M *et al.* Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. *Gut* 2006; **55**: 182–90.

13 Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun* 2011; **25**: 397–407.

14 Larsson MH, Miketa A, Martinez V. Lack of interaction between psychological stress and DSS-induced colitis affecting colonic sensitivity during colorectal distension in mice. *Stress* 2009; **12**: 434–44.

© 2013 John Wiley & Sons Ltd

15 Melgar S, Engstrom K, Jagervall A, Martinez V. Psychological stress reactivates dextran sulfate sodium-induced chronic colitis in mice. *Stress* 2008; **11**: 348–62.
16 Teran-Ventura E, Roca M, Martin MT, Abarca ML, Martinez V, Vergara P. Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats. *Microb Ecol* 2010; **60**: 691–702.
17 Salzman NH, de Jong H, Paterson Y, Harmsen HJ, Welling GW, Bos NA. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. *Microbiology* 2002; **148**: 3651–60.
18 Selinummi J, Seppala J, Yli-Harja O, Puhakka JA. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. *Biotechniques* 2005; **39**: 859–63.
19 Pelissier MA, Vasquez N, Balamurugan R *et al.* Metronidazole effects on microbiota and mucus layer thickness in the rat gut. *FEMS Microbiol Ecol* 2010; **73**: 601–10.
20 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402–8.
21 Swidsinski A, Loening-Baucke V, Theissig F *et al.* Comparative study of the intestinal mucus barrier in normal and inflamed colon. *Gut* 2007; **56**: 343–50.
22 Amador-Arjona A, Delgado-Morales R, Belda X *et al.* Susceptibility to stress in transgenic mice overexpressing TrkC, a model of panic disorder. *J Psychiatr Res* 2010; **44**: 157–67.
23 Lupp C, Robertson ML, Wickham ME *et al.* Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2007; **2**: 204.
24 Swidsinski A, Sydora BC, Doerffel Y *et al.* Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. *Inflamm Bowel Dis* 2007; **13**: 963–70.
25 Zoetendal EG, Mackie RI. Molecular methods in microbial ecology. In: Tannock GW, ed. *Probiotics and Prebiotics: Scientific Aspects*, 1st edn. Dunedin, New Zealand: Caister Academic Press, University of Otago, 2005: 1–24.
26 Larauche M, Gourcerol G, Million M, Adelson DW, Tache Y. Repeated psychological stress-induced alterations of visceral sensitivity and colonic motor functions in mice: influence of surgery and postoperative single housing on visceromotor responses. *Stress* 2010; **13**: 343–54.
27 Derrien M, Collado MC, Ben-Amor K, Salminen S, de Vos WM. The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract. *Appl Environ Microbiol* 2008; **74**: 1646–8.
28 Joossens M, Huys G, Cnockaert M *et al.* Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* 2011; **60**: 631–7.
29 O'Malley D, Julio-Pieper M, Gibney SM, Dinan TG, Cryan JF. Distinct alterations in colonic morphology and physiology in two rat models of enhanced stress-induced anxiety and depression-like behaviour. *Stress* 2010; **13**: 114–22.
30 Martinez V, Wang L, Rivier J, Grigoriadis D, Tache Y. Central CRF urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. *J Physiol* 2004; **556**: 221–34.
31 Soderholm JD, Perdue MH. Stress and gastrointestinal tract. II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G7–13.
32 Caso JR, Leza JC, Menchen L. The effects of physical and psychological stress on the gastro-intestinal tract: lessons from animal models. *Curr Mol Med* 2008; **8**: 299–312.
33 Bergstrom KS, Guttman JA, Rumi M *et al.* Modulation of intestinal goblet cell function during infection by an attaching and effacing bacterial pathogen. *Infect Immun* 2008; **76**: 796–811.
34 Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S. The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. *FEMS Microbiol Rev* 2011; **35**: 681–704.
35 Salzman NH. Microbiota-immune system interaction: an uneasy alliance. *Curr Opin Microbiol* 2011; **14**: 99–105.
36 Bishara J, Peled N, Pitlik S, Samra Z. Mortality of patients with antibiotic-associated diarrhoea: the impact of Clostridium difficile. *J Hosp Infect* 2008; **68**: 308–14.
37 Buffie CG, Jarchum I, Equinda M *et al.* Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. *Infect Immun* 2012; **80**: 62–73.
38 Chang JY, Antonopoulos DA, Kalra A *et al.* Decreased diversity of the fecal Microbiome in recurrent Clostridium difficile-associated diarrhea. *J Infect Dis* 2008; **197**: 435–8.
39 Videla S, Vilaseca J, Guarner F *et al.* Role of intestinal microflora in chronic inflammation and ulceration of the rat colon. *Gut* 1994; **35**: 1090–7.
40 Schultsz C, Van Den Berg FM, Ten Kate FW, Tytgat GN, Dankert J. The intestinal mucus layer from patients with inflammatory bowel disease harbors high numbers of bacteria compared with controls. *Gastroenterology* 1999; **117**: 1089–97.
41 Soderholm JD, Yang PC, Ceponis P *et al.* Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 2002; **123**: 1099–108.
42 Guarner F, Malagelada JR. Role of bacteria in experimental colitis. *Best Pract Res Clin Gastroenterol* 2003; **17**: 793–804.
43 Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. *Gut Microbes* 2011; **2**: 145–58.
44 Tlaskalova-Hogenova H, Stepankova R, Kozakova H *et al.* The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. *Cell Mol Immunol* 2011; **8**: 110–20.
45 Martinez-Medina M, Aldeguer X, Gonzalez-Huix F, Acero D, Garcia-Gil LJ. Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm Bowel Dis* 2006; **12**: 1136–45.

 © 2013 John Wiley & Sons Ltd

46 Sudo N, Chida Y, Aiba Y *et al.* Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* 2004; **558**: 263–75.
47 Bravo JA, Forsythe P, Chew MV *et al.* Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* 2011; **108**: 16050–5.
48 Gareau MG, Wine E, Rodrigues DM *et al.* Bacterial infection causes stress-induced memory dysfunction in mice. *Gut* 2011; **60**: 307–17.
49 Rousseaux C, Thuru X, Gelot A *et al.* Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat Med* 2007; **13**: 35–7.
50 Amaral FA, Sachs D, Costa VV *et al.* Commensal microbiota is fundamental for the development of inflammatory pain. *Proc Natl Acad Sci USA* 2008; **105**: 2193–7.
51 Brusberg M, Arvidsson S, Kang D, Larsson H, Lindstrom E, Martinez V. CB1 receptors mediate the analgesic effects of cannabinoids on colorectal distension-induced visceral pain in rodents. *J Neurosci* 2009; **29**: 1554–64.
52 Cremon C, Carini G, Wang B *et al.* Intestinal serotonin release, sensory neuron activation, and abdominal pain in irritable bowel syndrome. *Am J Gastroenterol* 2011; **106**: 1290–8.
53 Yuce B, Kemmer M, Qian G *et al.* Cannabinoid 1 receptors modulate intestinal sensory and motor function in rat. *Neurogastroenterol Motil* 2010; **22**: 672–e205.
54 Zoppi S, Madrigal JL, Perez-Nievas BG *et al.* Endogenous cannabinoid system regulates intestinal barrier function *in vivo* through cannabinoid type 1 receptor activation. *Am J Physiol Gastrointest Liver Physiol* 2011; **302**: G565–71.
55 Muccioli GG, Naslain D, Backhed F *et al.* The endocannabinoid system links gut microbiota to adipogenesis. *Mol Syst Biol* 2010; **6**: 392.
56 Julio-Pieper M, O'Mahony CM, Clarke G, Bravo JA, Dinan TG, Cryan JF. Chronic stress-induced alterations in mouse colonic 5-HT and defecation responses are strain dependent. *Stress* 2012; **15**: 218–26.
57 Bertrand PP, Barajas-Espinosa A, Neshat S, Bertrand RL, Lomax AE. Analysis of real-time serotonin (5-HT) availability during experimental colitis in mouse. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G446–55.
58 Wheatcroft J, Wakelin D, Smith A, Mahoney CR, Mawe G, Spiller R. Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction. *Neurogastroenterol Motil* 2005; **17**: 863–70.
59 Bradesi S, Martinez V, Lao L, Larsson H, Mayer EA. Involvement of vasopressin 3 receptors in chronic psychological stress-induced visceral hyper algesia in rats. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G302–9.
60 van den Wijngaard RM, Welting O, Bulmer DC *et al.* Possible role for TRPV1 in neomycin-induced inhibition of visceral hypersensitivity in rat. *Neurogastroenterol Motil* 2009; **21**: 863–e60.
61 Reber SO. Stress and animal models of inflammatory bowel disease–an update on the role of the hypothalamo-pituitary-adrenal axis. *Psychoneuroendocrinology* 2012; **37**: 1–19.
62 Santos J, Yang PC, Soderholm JD, Benjamin M, Perdue MH. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. *Gut* 2001; **48**: 630–6.
63 Tsuruta T, Inoue R, Iwanaga T, Hara H, Yajima T. Development of a method for the identification of S-IgA-coated bacterial composition in mouse and human feces. *Biosci Biotechnol Biochem* 2010; **74**: 968–73.
64 Mathias A, Corthesy B. N-Glycans on secretory component: mediators of the interaction between secretory IgA and gram-positive commensals sustaining intestinal homeostasis. *Gut Microbes* 2011; **2**: 287–93.
65 Duerkop BA, Vaishnava S, Hooper LV. Immune responses to the microbiota at the intestinal mucosal surface. *Immunity* 2009; **31**: 368–76.

© 2013 John Wiley & Sons Ltd

Microb Ecol (2013) 66:232–243
DOI 10.1007/s00248-013-0241-0

HOST MICROBE INTERACTIONS

Environment-Related Adaptive Changes of Gut Commensal Microbiota Do not Alter Colonic Toll-Like Receptors but Modulate the Local Expression of Sensory-Related Systems in Rats

M. Aguilera · P. Vergara · V. Martínez

Received: 19 December 2012 / Accepted: 26 April 2013 / Published online: 12 May 2013
© Springer Science+Business Media New York 2013

Abstract Pathogenic and protective roles have been attributed to gut commensal microbiota (GCM) in gastrointestinal inflammatory and functional disorders. We have shown that the adaptation to a new environment implies specific changes in the composition of GCM. Here we assessed if environment-related adaptive changes of GCM modulate the expression of colonic Toll-like receptors (TLRs) and sensory-related systems in rats. Adult male SD rats were maintained under different environmental conditions: barrier-breed-and-maintained, barrier-breed adapted to conventional conditions or conventional-breed-and-maintained. Fluorescent in situ hybridization and real-time quantitative PCR (qPCR) were used to characterize luminal ceco-colonic microbiota. Colonic expression of TLR2, TLR4, TLR5, and TLR7, cannabinoid receptors (CB1/CB2), μ-opioid receptor (MOR), transient receptor potential vanilloid (TRPV1, TRPV3, and TRPV4), protease-activated receptor 2 (PAR-2), and calcitonin gene-related peptide were quantified by RT-qPCR. CB1, CB2 and MOR expression, was evaluated also by immunohistochemistry. In rats, housing-related environmental conditions induce specific changes of GCM, without impact on the expression of TLR-dependent bacterial recognition systems. Expression of sensory-related markers (MOR, TRPV3, PAR-2, and CB2) decreased with the adaptation to a conventional environment, correlating with changes in *Bacteroides* spp., *Lactobacillus* spp., and *Bifidobacterium* spp. counts. This suggests an interaction between GCM and visceral sensory mechanisms, which might be part of the mechanisms underlying the beneficial effects of some bacterial groups on functional and inflammatory gastrointestinal disorders.

M. Aguilera · P. Vergara · V. Martínez
Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain

P. Vergara · V. Martínez
Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Madrid, Spain

M. Aguilera · P. Vergara · V. Martínez
Neuroscience Institute, Universitat Autònoma de Barcelona, Barcelona, Spain

V. Martínez (✉)
Edifici V, Unitat de Fisiologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
e-mail: vicente.martinez@uab.es

Introduction

Gut commensal microbiota (GCM) is a dynamic microbiological system comprised by a large number of bacterial species [6]. Numerous evidences support a critical role for commensal bacteria in the maintenance of gut homeostasis. For instance, GCM appears to be of crucial importance in the modulation of local immune responses [23] and, through these mechanisms, to be a pathogenic component of inflammatory and functional gastrointestinal alterations, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Indeed several reports suggest that GCM composition is altered in both IBD and IBS patients [10, 25, 39]. Therefore, modulation of the commensal microbiota, mainly through the administration of probiotics and/or prebiotics, has become an attractive, and largely explored, approach for the treatment of these alterations. This is based on numerous studies, both in animals and humans, suggesting that specific microbial agents might modulate immune responses, local epithelial functions, motor activity, afferent

sensory information associated to visceral sensitivity, and even central nervous system responses associated to intestinal stimuli [10, 14–16, 20]. However, the mechanisms mediating these effects remain largely unknown.

Recent data derived from animal models of IBS evaluating sensory information arising from the gut suggest that GCM might influence neuronal and/or neuro-immune mechanisms within the gut [10]. In mice and rats, modifications of the intestinal commensal microbiota result in changes in nociceptive responses that have been associated to alterations in neuro-immune mediators implicated in sensory mechanisms, including pain responses, within the gut [29, 33]. We have recently shown that GCM has spontaneous, environmental-related, adaptive changes. In particular, animals breed under barrier conditions, when moved to standard housing conditions, present an adaptive shift in their GCM towards the characteristic of the new environment [31]. Similar environmental-related changes have been also described in mice [7, 26]. The significance of these changes is not clear but might implicate alterations in gut homeostasis, leading to altered functional responses and/or the development of pathophysiological states.

Based on these observations, we aimed to correlate environmental-related adaptive changes in GCM with changes in neuro-immune sensory systems within the gut. For this, we characterized spontaneous changes of ceco-colonic commensal microbiota, using fluorescence in situ hybridization (FISH), in rats born and bred under different environmental conditions. FISH results were confirmed by real-time quantitative PCR (qPCR). In the same animals the gene expression of cannabinoid receptors (CB1 and CB2), transient receptor potential vanilloid channels (TRPV1, TRPV3 and TRPV4), protease-activated receptor 2 (PAR-2), μ-opioid receptors (MOR) and calcitonine-gene related peptide (CGRP) was assessed also by RT-qPCR. Moreover, the intestinal expression of cannabinoid receptors (CB1 and CB2) and MOR was also evaluated by immunohistochemistry. All of these receptors/mediators participate in sensory and secretomotor responses within the gut and have been implicated in both inflammatory and functional gastrointestinal alterations [3–5, 12, 21].

The interaction between gut microbiota and the host is known to be mediated through pattern recognition receptors (PRR). Toll-like receptors (TLRs) are among the best characterized PRRs; they are largely expressed within the gut and are key components mediating bacteria–host interactions and microbial recognition [8, 9, 27, 28]. Therefore, we also assessed if environmental-related changes in the luminal GCM affect the local expression of TLRs, specifically the TLR subtypes 2, 4, 5, and 7 (TLR2, TLR4, TLR5, and TLR7).

Materials and Methods

Animals

Four 6-week-old and four 9-week-old male OFA Sprague–Dawley rats (specific pathogen free) bred and maintained in a barrier-protected area with all materials, water, food, and bedding sterilized before entering the barrier were obtained from Charles River Laboratories (Lyon, France). The original microbiota inoculated in these animals (as provided by the breeder) consisted of *Bacteroides distasonis*, *Lactobacillus acidophilus*, *Lactobacillus salivaris*, Schaedler fusiform-shaped bacterium, three strains of CRL fusiform-shaped bacterium, CRL mouse spirochete, *Escherichia coli* (non-hemolytic), *Streptococcus faecalis* (group D), and *Enterococcus* spp. In addition, four 9-week-old male OFA Sprague–Dawley rats bred in conventional conditions in the Animal Facility of the Universitat Autònoma de Barcelona were used. This conventional colony was established in 1994 from OFA Sprague–Dawley rats from Charles River Laboratories (Lyon, France) and has been appropriately cross-bred in order to maintain genetic stability. When in conventional conditions, water, food, and bedding were given to animals as facilitated by the commercial provider, without any further treatment.

All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya.

Experimental Groups and Sample Collection

Three experimental groups were defined: (1) 9-week-old barrier-bred rats (barrier-breed-and-maintained group; $n=4$), (2) 9-week-old rats born and bred under conventional conditions (conventional-breed-and-maintained group; $n=4$), and (3) 6-week-old barrier rats maintained under conventional conditions at the animal facility of the UAB for 3 weeks (barrier-breed adapted to conventional conditions group; $n=4$). All animals were 9 weeks old at the time of testing. To avoid potential time-related changes in microbiota, all experimental groups were processed simultaneously.

Animals were euthanized by CO_2 inhalation followed by a thoracotomy. Thereafter, the abdominal cavity was opened, the ceco-colonic region was localized, and fecal content and tissue samples were collected and frozen immediately with liquid nitrogen. All samples were stored at −80 °C until analysis. For immunohistochemistry analysis, a 3-cm segment of the mid-portion of the colon was removed and fixed overnight with 4 % paraformaldehyde.

Enumeration of Bacteria Using Fluorescence In Situ Hybridization

For FISH, general methods previously reported were followed [18, 31]. Oligonucleotide probes consisted of a single-strain DNA covalently linked with Cy3 at the 5′ end (see Table 1 for details of the probes used). All probes were obtained from Tib MolBiol or Biomers.

Frozen fecal contents (about 0.5 g) were thawed, and 4.5 ml of Millipore filtered phosphate-buffered saline (PBS) 1×, at pH 7.2, and three to five glass beads (3 mm in diameter) were added and homogenized on a vortex for 3 min. The suspension obtained was then centrifuged for 1 min at 700 g. One milliliter of the supernatant was collected and fixed overnight (4 °C) in 3 ml of freshly prepared 4 % paraformaldehyde and stored at −20 °C until analyzed.

At the time of analysis, samples were diluted in PBS 1× and spotted on pre-cleaned, gelatin-coated [0.1 % gelatin, 0.01 % $KCr(SO_4)_2$] Teflon-printed slides (ten wells, diameter 8 mm; K-11, Knittel Gläss), dried at room temperature, and dehydrated in 96 % ethanol (10 min). Samples were hybridized in a dark moist chamber by addition of 100 µl hybridization buffer (20 mM Tris–HCl, 0.9 M NaCl, 0.1 % SDS at pH 7.2) preheated with the corresponding Cy3-labeled oligonucleotide probe (with a final concentration of 10 ng/µl) in each well. Hybridization temperatures and duration were used as previously described to achieve optimal stringency [31]. Lysozyme treatment (37 °C, 90 min) was performed prior to the hybridization process for detection of *Lactobacillus* spp.

After hybridization, the slides were rinsed in a pre-warmed washing buffer (20 mM Tris–HCl, 0.9 M NaCl at pH 7.2) for 30 min, at each hybridization temperature, and then cleaned with miliQ water to remove unbound probes. Washed slides were air-dried and mounted with Vectashield® Mounting Media with-DAPI (H-1200; Vector Laboratories) to stain the chromosomes as a control signal.

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; Carl Zeiss Microscopy). For quantification of bacteria, 20 randomly selected fields were photographed and the number of hybridized cells counted using the CellC software [30].

Relative Quantification of Bacteria Using Real-Time Quantitative PCR

Total DNA was isolated from frozen ceco-colonic content using QIAamp® DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions. Thereafter, DNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), diluted to equal concentrations with sterile deionised water, and stored at −20 °C until analysis.

Table 1 Primers (FISH) and probes (qPCR) used for quantification of bacterial 16S rRNA genes

	FISH			RT-qPCR		
	Probe	Cy3 5′–3′	Ref.		FAM 5′–3′	Ref.
Non-bacteria	NON 338	ACATCCTACGGGAGGC	[18, 31]			
Total bacteria	EUB 338	GCTGCCTCCCGTAGGAGT	[18, 31]			
Enterobacteria	ENT-D	TGCTCTCGCGAGG TCGCTTCTCTT	[18, 31]			
Bacteroides spp	BAC 303	CAATGTGGG GGACCTT	[18, 31]	Forward Reverse	AG AGG AAG GTC CCC GC TAC TTG GCT GGT T	[40]
				Probe	CA TTG ACC AAT ATT CCT CAC TGC TGC	
Bifidobacterium spp	BIF 164	CATCCGGCAT TACCACCC	[18, 31]	Forward Reverse	CG TGC TTA ACA CAT GCA A CAC CCG TTT CCA GGA G	[40]
				Probe	TCA CGC ATT ACT CAC CCG TTC G	
Clostridium cluster XIVa	EREC 482	GCTTCTTAGTC AGGTACCG	[18, 31]	Forward Reverse	A GTG GGG AAT ATT T TGA GTT TCA TTC TTG C	[40]
				Probe	AATGACGGTACCTGA	
Lactobacillus spp	LAB 158	GGTATTAGCAC CTGTTTCCA	[18, 31]	Forward Reverse	TGG ATG CCT TGG CAC TAG GA AAA TCT CCG GAT CA A AGC TTA CTT AT	[17]
				Probe	TAT TAG TTC CGT CCT TCA TC	

The relative abundance of bacteria was measured using 16S rRNA gene-targeting hydrolysis probes (Custom TaqMan assays; Applied Biosystems; see Table 1 for details of the different probes used). Amplifications were carried out in a final volume of 20 μL [1 μL of assay (primers 18 μM and probe 5 μM), 10 μL of TaqMan Universal Master Mix II, 5 μL of miliQ water, and 4 μL of DNA (40 ng for *Bifidobacterium* spp., *Clostridium* spp., and *Bacteroides* spp. and 4 ng for *Lactobacillus* spp.)]. Amplification program was 95 °C during 10 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. For *Lactobacillus* spp., the amplification program was increased to 50 cycles. All samples and the negative controls were assayed for triplicate. The barrier-breed-and-maintained group served as the calibrator. To determine the relative bacterial content, an expression value of 1 was assigned to the barrier-breed-and-maintained group, and expression levels in the other groups were referred to it. In these conditions, relative expression values above 1 imply an increase in bacterial counts in relation to the barrier-breed-and-maintained group. Conversely, relative expression values below 1 imply a decrease in bacterial counts in relation to the barrier-breed-and-maintained group.

Gene Expression Using Quantitative Reverse Transcription-PCR

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit (AM1924; Ambion/Applied Biosystems); thereafter, a two-step RT-PCR was performed. RNA samples were converted to cDNA using a high-capacity cDNA reverse transcription kit (4368814; Applied Biosystems). Only a consistent 260/280 ratio (between 1.8 and 2) found with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was accepted to perform quantitative real-time PCR using hydrolysis probes. TaqMan gene expression assays for CB1 receptors (Rn00562880_m1), CB2 receptors (Rn00571953_m1), MOR (Rn01430371_m1), TRPV1 (Rn00583117_m1), TRPV3 (Rn01460303_m1), TRPV4 (Rn00576745_m1), PAR-2 (Rn00588089_m1), CGRP (Rn01511354_m1), TLR2 (Rn02133647_s1), TLR4 (Rn00569848_m1), TLR5 (Rn01411671_s1), and TLR7 (Rn01771083_s1) were used (Applied Biosystems). β-Actin (Rn00667869_m1) was used as endogenous housekeeping and the barrier-breed-and-maintained group served as the calibrator.

The PCR reaction mixture was transferred to a MicroAmp optical 96-well reaction plate and incubated on the ABI 7900 HT Sequence Detection System (Applied Biosystems). RQ Manager 1.2 software was used to obtain the cycle threshold for each sample; thereafter, all data were analyzed with the comparative Ct method ($2^{-\Delta\Delta Ct}$) [24]. All samples and the negative controls were assayed for triplicate.

Immunohistochemistry

Paraffin-embedded tissue sections (5 μm in thickness) were deparaffinized and rehydrated with a battery gradient of alcohols. Antigen retrieval for CB1 receptor and MOR was achieved by processing the slides in a microwave with 10 mM of citrate solution. Epitope retrieval for CB2 receptor was performed using a pressure cooker (at full pressure, for 3 min) in Tris–EDTA solution buffer. Quenching of endogenous peroxidase was performed by 1 h of incubation with 5 % H_2O_2 in distilled water. Primary antibodies included a rabbit polyclonal anti-CB1 (1:100; rabbit polyclonal to cannabinoid receptor 1, ab23703; Abcam), a rabbit polyclonal anti-CB2 [1:100; rabbit polyclonal to cannabinoid receptor 2 (H-60), sc-25494; Santa Cruz Biotechnology], and a rabbit polyclonal anti-MOR (1:2,500; rabbit polyclonal to mu opioid receptor AB1580; Chemicon/Millipore). The secondary antibody used was a biotinylated polyclonal swine anti-rabbit IgG (E 0353; DakoCytomation). Detection was performed with avidin/peroxidase kit (Vectastain Elite ABC kit, PK-6100; Vector Laboratories); antigen–antibody complexes were reveled with 3-3′-diaminobenzidine (SK-4100 DAB; Vector Laboratories), with the same time exposure per antibody, and sections were counterstained with hematoxylin. Specificity of staining was confirmed by omission of the primary antibody.

Quantification of Immune-Positive Signal in the Myenteric Plexus

For CB2, immunopositive cells were counted in 30, randomly selected, myenteric ganglia, in duplicate, for each tissue sample. Cells were considered to be immunopositive if they expressed more labeling than the background levels seen in the negative controls.

MOR immunoreactive myenteric ganglia were quantified by means of gray density using ImageJ software (NIH Image, USA). A minimum of 15 myenteric ganglia per sample were evaluated. Images were taken with the same light intensity, and control negative signals were used to determine the positive gray threshold. All counting was performed on coded slides to avoid any bias.

Statistical Analysis

Data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for qPCR data. Comparison between multiple groups were performed using one-way analysis of variance (one-way ANOVA), followed when necessary by Student–Newman–Keuls multiple comparisons test. Correlation between parameters was assessed by linear regression and Pearson's analysis. In all cases, results were considered

as statistically significant when $P<0.05$. All statistical analysis and graphs were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

Results

Characterization of Intestinal (Ceco-colonic) Commensal Microbiota by FISH

Total mean bacterial counts, as determined using DAPI staining and EUB 338 hybridization, oscillated between 5.3×10^9 and 2.2×10^{10} cells/ml and were comparable in all experimental groups and within the margins previously described [13, 31]. However, FISH analysis revealed significant differences among groups in the commensal microbiota composition (Table 2; Figs. 1 and 2).

Among Gram-negative bacteria, the counts of Enterobacteriaceae (ENT-D probe) were scarce (in many cases, less than 1 % of the flora quantified), regardless of the group considered. In contrast, *Bacteroides* spp. group (BAC 303 probe) was relatively abundant, and counts were significantly higher in the barrier-breed-and-maintained and the conventional-breed-and-maintained groups when compared with the barrier-breed adapted to conventional conditions group (Table 2; Figs. 1 and 2).

Overall, Gram-positive bacteria represented more than 80 % of the total FISH-quantified microbiota. In particular, *Clostridium coccoides–Eubacterium rectale* group (*Clostridium* cluster XIVa, EREC 482 probe) accounted for the largest bacterial population in all experimental groups. Counts for *Lactobacillus* spp. and *Enterococcus* spp. (LAB 158 probe) and *Bifidobacterium* spp. (BIF 164 probe) were higher in fecal samples from the conventional-breed-and-maintained group when compared with the barrier-breed-and-maintained and barrier-breed adapted to conventional conditions groups, which showed clear transitional changes towards adaptation to conventional conditions (Table 2; Figs. 1 and 2).

Characterization of Intestinal (Ceco-colonic) Commensal Microbiota by qPCR

qPCR was used to confirm FISH results. The bacterial groups assessed (*Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Clostridium* cluster XIVa) were detected in all fecal samples. However, relative expression levels (indicative of the bacterial density) were different among bacterial and among experimental groups. Overall, the relative abundance for the different bacterial groups followed the same pattern obtained using FISH (Fig. 2), revealing the same changes associated to the environmental conditions.

Colonic Expression of TLRs

TLRs expression was similar across groups, regardless of the environmental conditions considered. Overall, colonic expression of TLR4 and TLR 5 was similar across groups and higher (by six-fold) than the expression of TLR2 or TLR7. Relative expression levels were TLR4 ~ TLR5 > TLR2 ~ TLR7 (Fig. 3).

Colonic Expression of Sensory-Related Markers and Correlation with Bacterial Counts

The most abundantly expressed sensory-related marker within the colon was PAR-2, with a minimum of ten-fold higher expression than other markers assessed, regardless of the experimental group considered. In all cases, CGRP

Table 2 Bacterial counts in the different experimental groups as determined by FISH

	Barrier-breed-and-maintained ($\times10^8$ cells/ml)	Barrier-breed adapted to conventional conditions ($\times10^8$ cells/ml)	Conventional-breed-and-maintained ($\times10^8$ cells/ml)
Total cells[a]	171.1±21.5	113.1±34.9	138.0±37.4
Enterobacteria	0.7±0.09	0.7±0.3	1.28±0.2
Bacteroides spp.	6.4±2.2	0.428±0.84[&]	2.77±0.7
Bifidobacterium spp.	0.2±0.06	0.27±0.17	1.8±0.6
Clostridium cluster XIVa	35.7±11.2	24.8±5.4	45.3±20
Lactobacillus spp.	0.3±0.1**	1.28±0.38*	3.4±0.8

Data are mean ± SEM, $n=4$ per group. Because of technical problems, one animal of the barrier-breed-and-maintained group was not analyzed for *Bifidobacterium* spp

[a] Total fecal microbial counts as determined by DAPI staining

*$P<0.05$ vs. conventional-breed-and-maintained group; **$P<0.01$ vs. conventional-breed-and-maintained group; [&] $P<0.05$ vs. barrier-breed-and-maintained group (ANOVA)

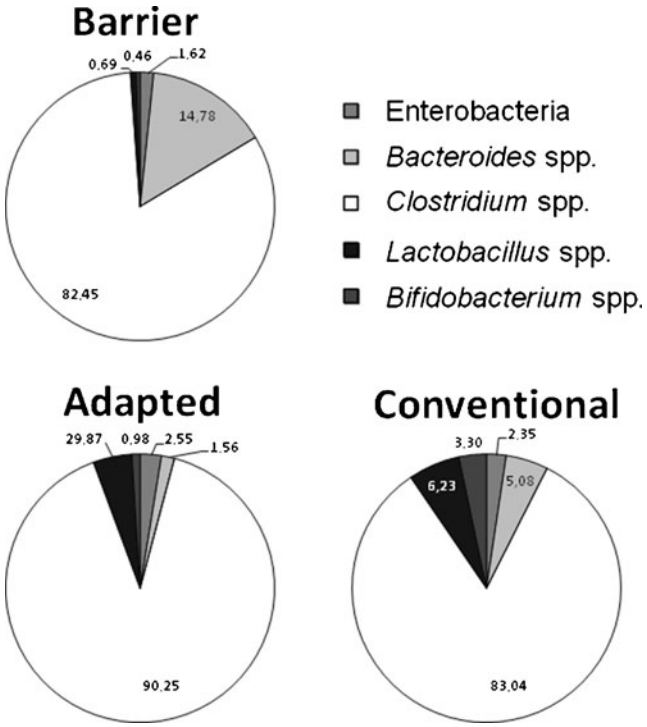


Figure 1 Relative composition of the ceco-colonic microbiota, as quantified by FISH, in the different experimental groups. Data represent relative abundance (%) of the different bacterial groups characterized by FISH (*Bacteroides* spp., Enterobacteriaceae, *Bifidobacterium* spp., *Lactobacillus–Enterococcus* spp., and *Clostridium* cluster XIVa group). Relative percent composition was calculated taking as 100 % the total counts of the bacterial groups assessed. *Barrier* barrier-breed-and-maintained group, *Adapted* barrier-breed adapted to conventional conditions group, *Conventional* conventional-breed-and-maintained group (see Table 1 for exact cell counts)

was undetectable. Other markers assessed, although detected in all samples analyzed, showed, in general, low levels of expression.

Expression of CB2 (P=0.1), PAR-2 (P=0.07), TRPV3 (P=0.03), and MOR (P=0.01) was higher in the barrier-breed-and-maintained group when compared to the conventional-breed-and-maintained group, showing intermediate levels of expression in the barrier-breed adapted to conventional conditions group (Fig. 4). Expression levels of CB1 and TRPV1 and 4 were similar across groups.

Expression levels of MOR and TRPV3 correlated with changes in the composition of the luminal microbiota. In particular, expression levels of TRPV3 showed a negative correlation with the counts of *Bifidobacterium* spp. (P=0.01; r^2=0.55), and *Lactobacillus* spp. (P=0.02; r^2=0.45) (Fig. 5). Similarly, Enterobacteria counts also tended to correlate in a negative manner with TRPV3 mRNA (P=0.07; r^2=0.27). On the other hand, expression levels of MOR showed a clear tendency to positively correlate with the counts of *Bacteroides* spp. (P=0.07; r^2=0.28) and to negatively correlate with *Lactobacillus* spp. counts (P=0.07; r^2= 0.27; Fig. 5). Similar correlations were determined when using relative bacterial abundance determined by qPCR (data not shown).

Localization and Quantification of Cannabinoid and μ Opioid Receptors by Immunohistochemistry

CB2 immunoreactivity was observed in the luminal surface of the epithelial microvilli and within ganglionic cells of the myenteric plexus. Very few immunoreactive cells were observed in the submucous plexus. Intense CB2 immunoreactivity was observed in Peyer's patches and also in some unidentified cells distributed through the submucosa, likely corresponding to immune cells. The smooth muscle of the blood vessels walls showed intense immunoreactivity. Overall, similar patterns of distribution and staining intensities were observed in all experimental groups (Fig. 6). Nevertheless, CB2-immunoreactive ganglionic cell density within the myenteric plexus had a clear trend to be lower in the conventional-breed-and-maintained group compared with the other experimental groups, although statistical significance was not achieved (P=0.053; Fig. 6).

Immunostaining for CB1 receptors was found in epithelial cells, mainly within the apical surface of the microvilli. In the submucosa, a fainter positive staining was seen in the smooth muscle of the blood vessel walls. No staining was observed in the muscle layers. Cells within ganglia of the myenteric plexus also appeared immunostained, while within the submucous plexus immunoreactive cells were rarely seen. Within the myenteric plexus, the density of immunoreactive cells was low: CB1–immunoreactive cells oscillated between zero and two cells/ganglion and was similar among all experimental groups (data not shown). Peyer's patches showed moderate staining in well-defined cells. Similar distribution and staining intensities were observed in all experimental groups (data not shown).

MOR immunoreactivity was mainly found within the ganglia of both myenteric and submucosal plexuses. Some epithelial cells as well as unidentified cells in the lamina propia also appeared stained (Fig. 6). Immunostaining intensity within the myenteric plexus was higher in the barrier-breed-and-maintained group and showed a clear tendency to decrease with the adaptation to conventional housing conditions (Fig. 6).

In all cases, absence of the primary antibody resulted in a complete loss of immunoreactivity, thus confirming the specificity of staining.

Discussion

During the recent years, there has been an increasing interest in the role of GCM in the maintenance of gastrointestinal homeostasis and its potential implication in pathophysiological

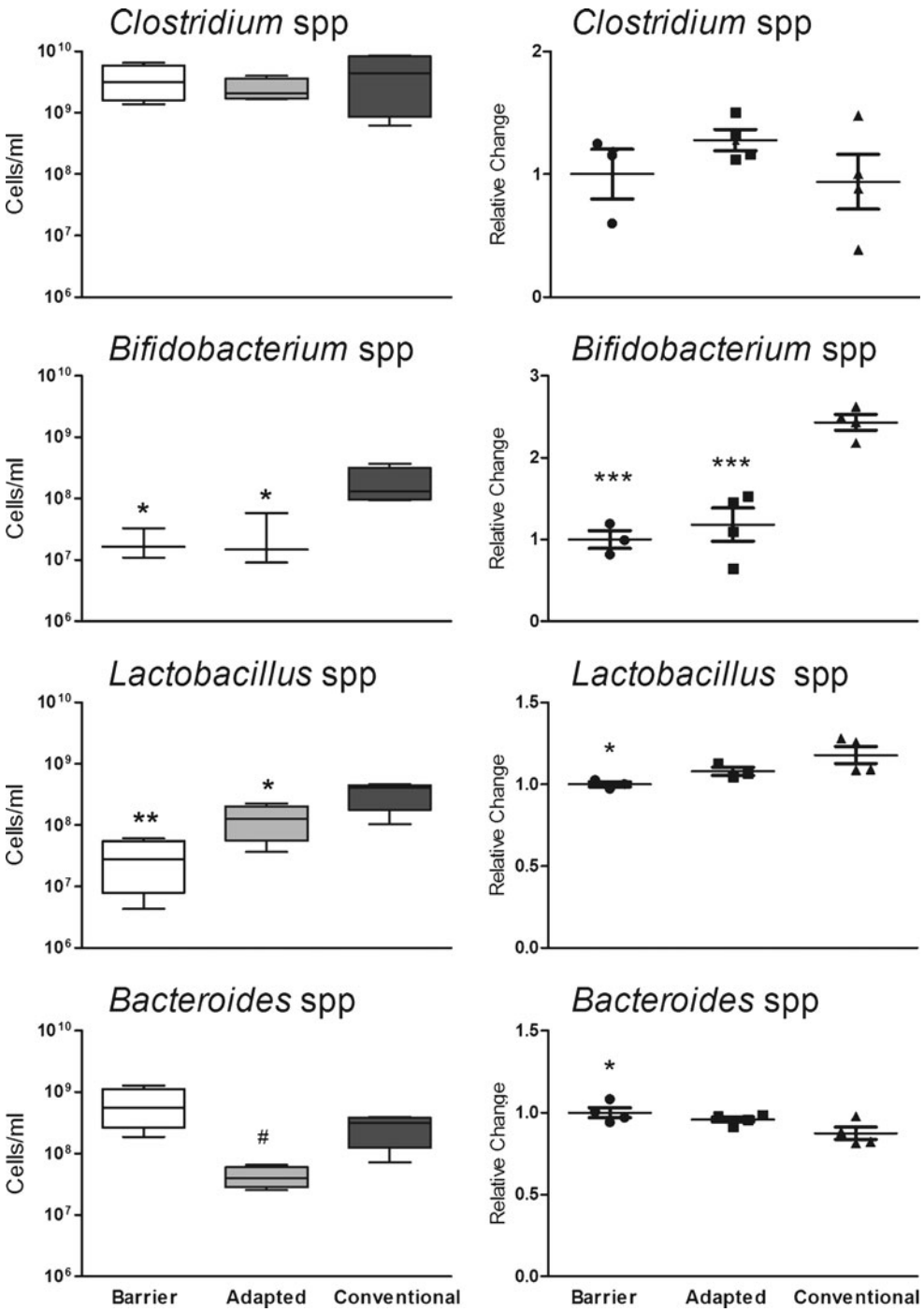


Figure 2 Comparison of the ceco-colonic microbiota, as quantified by FISH (*left column*) and qPCR (*right column*), in the same animals. FISH quantification (*left column*): data are media (interquartile range) ± SD for each bacterial group analyzed (n=4 per group). Because of technical problem, one animal of the barrier group was not analyzed for *Bifidobacterium* spp. *P<0.05 vs. the conventional group; **P<0.01 vs. the conventional group; $^{\#}P$<0.05 vs. the barrier group. qPCR quantification (*right column*): each point represents an individual animal. The *horizontal line* with errors represent the mean ± SEM (n=4 per group). Because of technical problems, one animal of the barrier group was not analyzed for *Bifidobacterium* spp. and *Clostridium* spp. and one animal of the adapted group was not analyzed for *Bacteroides* spp. *P<0.05 vs. the conventional group; ***P<0.001 vs. the conventional group. *Barrier* barrier-breed-and-maintained group, *Adapted* barrier-breed adapted to conventional conditions group, *Conventional* conventional-breed-and-maintained group

conditions. Here we present, for the first time, evidence that spontaneous variations in gut microbiota, related to environmental adaptation, are associated with changes in the expression of secretomotor and sensory-related markers within the gut (mainly the opioid, vanilloid, and endocannabinoid systems), without alterations in TLRs expression.

We show that the environmental conditions of breeding/housing of the animals determine, at least partially, the composition of the GCM, as previously shown for both rats and mice [7, 26, 31]. Moreover, in agreement with our previous observations [31], results obtained indicate that the relative composition of the ceco-colonic microbiota in rats varies spontaneously with changes in the environmental conditions. Changes in the composition of GCM observed here are in concordance with those previously described by us in similar experimental conditions and using animals of the same genetic background [31]. Main microbial changes associated to the adaptation from a barrier to a conventional environment implied a loss of *Bacteroides* spp. with a

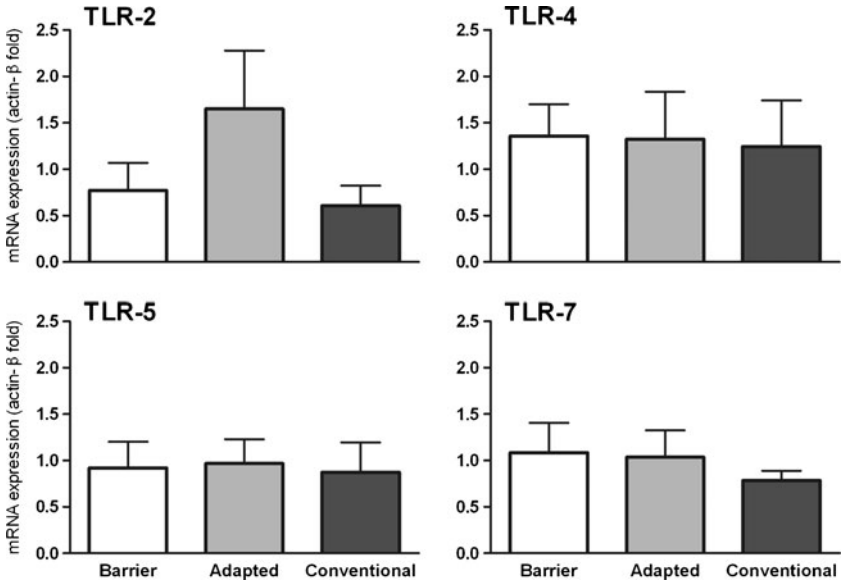


Figure 3 mRNA expression of TLR2, TLR4, TLR5, and TLR7 in colonic tissue samples of the different experimental groups. *Barrier* barrier-breed-and-maintained group, *Adapted* barrier-breed adapted to conventional conditions group, *Conventional* conventional-breed-and-maintained group. Data are mean ± SEM of four animals per group

simultaneous increase in the counts of *Lactobacillus–Enterococcus* spp. and *Bifidobacterium* spp. Quantitative changes in bacterial composition across groups were further confirmed using qPCR. Overall, relative bacterial abundances assessed by qPCR or direct bacterial quantifications by FISH were equivalent. Since qPCR is a more sensitive technique than FISH, these similarities reinforce the value of our FISH data and indicate that the results described reflect a consistent change in microbial composition.

Numerous reports have implicated GCM in the development of immune responses within the gut and identified bacterial flora as a significant component of the pathogenesis of several intestinal disorders, including inflammatory bowel disease and irritable bowel syndrome [10, 23, 25, 39]. Following these observations, several studies suggest that microbiota might influence immune- and neurally-mediated responses within the gut. In particular, several lines of evidence suggest that bacterial flora is implicated in the modulation of sensory mechanisms arising from the gut and, therefore, in changes in visceral pain-related responses. First, clinical and preclinical studies have shown that administration of certain probiotics can prevent abdominal symptoms in IBS and IBD patients (pain and discomfort, bloating, or altered bowel habits) [14, 16, 22, 32, 35–37]. Second, probiotic-like treatments reduced visceral pain-related responses in animal models of the disease [15, 29]. Despite the clinical interest of these observations, the mechanisms mediating these beneficial effects remain largely unknown. Animal data suggest, at least, a microbial-dependent modulation of the opioid and the endocannabinoid systems, both mediating analgesic responses within the gut [4, 12, 21], as a potential underlying mechanism [29, 33]. Here

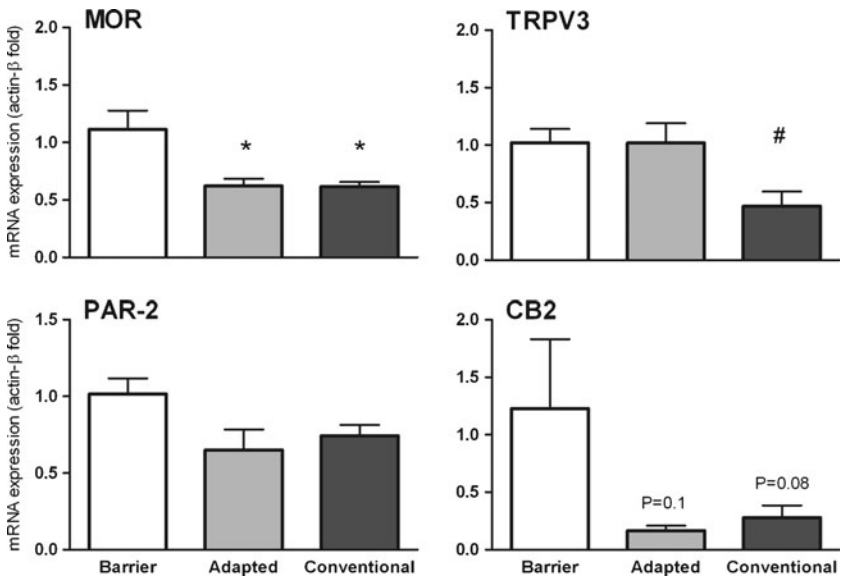


Figure 4 mRNA expression of MOR, TRPV3, PAR-2, and CB2 in colonic tissue samples of the different experimental groups. Data are mean ± SEM of four animals per group. *$P<0.05$ vs. barrier; #$P<0.05$ vs. other groups. *Barrier* barrier-breed-and-maintained group, *Adapted* barrier-breed adapted to conventional conditions group, *Conventional* conventional-breed-and-maintained group

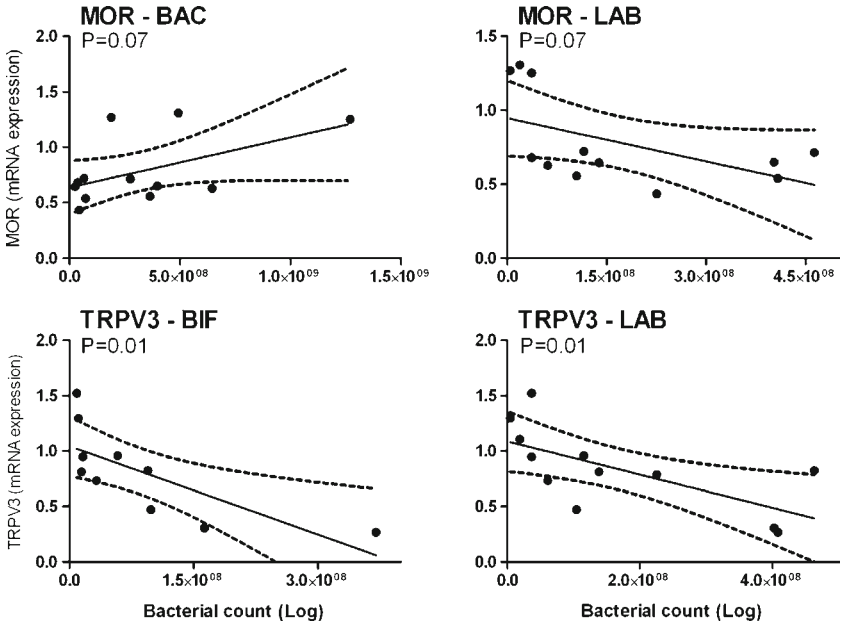


Figure 5 Correlation between the relative expression of sensory-related markers and bacterial counts. *Top* MOR; *bottom* TRPV3 for either *Bacteroides* spp. (*BAC*), *Lactobacillus–Enterococcus* spp. (*LAB*), or *Bifidobacterium* spp. (*BIF*). Each *point* represents an individual animal. Broken lines represent the 95 % confidence interval

we show that, in rats, spontaneous adaptive variations of GCM, associated to environmental changes, lead to a modulation of the expression of sensory-related systems within the gut, including the opioid, the endocannabinoid, and the vanilloid (TRPV) systems. Altogether these data support a role for microbiota affecting sensory-related gastrointestinal regulatory mechanisms.

Preliminary observations suggest that specific bacterial strains might modulate the endogenous expression of several mediators implicated in viscero-sensitivity. For instance, a specific strain of *L. acidophilus* given as a probiotic was able to modulate the content of CB2 and MOR in the gut and to reduce visceral pain responses in rats [29]. These results agree with the present observations showing that spontaneous adaptive variations of the GCM imply changes in the intestinal expression of receptors implicated in sensory/nociceptive-related mechanisms. In the present study, we did not correlate changes in receptor expression with any particular strain of bacteria, but with large spontaneous changes in gut commensal microbiota. From our observations, spontaneous fluctuations of *Bacteroides* spp., *Bifidobacterium* spp., and *Lactobacillus* spp. strains correlate with changes in sensory-related markers. Although no clear cause–effect relationship can be inferred, these observations suggest that such bacterial strains might be (directly or indirectly) implicated in the changes observed in sensory-related markers. Previous studies have pointed towards two main genera of bacteria as having beneficial effects in gut homeostasis, including the modulation of visceral pain-related responses: *Lactobacillus* spp. and *Bifidobacterium* spp. [14–16, 22, 29, 36, 37]. In our conditions, spontaneous changes in these bacterial groups correlated with variations in TRPV3 and MOR expression. This partially agrees with previous reports in which gut microbiota was related with the content of MOR and CB2 receptors in the gut, leading to an analgesia-like state [29]. Interestingly, expression changes were observed in both pro-nociceptive (TRPV3 and PAR-2) and anti-nociceptive (MOR and CB2) systems. In all cases, down-regulation was observed in the adaptation process from barrier to conventional conditions. This might suggest differences in pain responses between animals maintained in barrier or standard conditions or during the adaptation process from one environment to the other, although this remains to be demonstrated. Supporting this view, large alterations of gut microbiota, associated to the treatment with antibiotics or by adaptation to a standard, non-sterile environment, were associated to altered visceral pain responses in mice [1, 34]. Moreover, mice data suggest also that GCM is fundamental for the development of inflammatory pain [2]. Therefore, it is feasible to assume that microbial changes may result in alterations in visceral pain responses.

It is important to remind that receptor modulation was initially determined at the gene expression level (mRNA). To further determine if expression changes could have consequences at the protein level, we further assessed the expression of CB1/CB2 and MOR in colonic tissues using immunohistochemistry. In all cases CB1, CB2, and MOR immunoreactivity were identified throughout the colon. Tissue distribution observed for either receptor was in accordance to that previously described for the colon in mice, rats, and pigs [11, 19, 38]. Overall, no differences among groups in immunoreactivity were observed for none of the receptors assessed. Nevertheless, a more detailed analysis of immunoreactivity in the myenteric plexus indicated lower protein content in ganglionic structures in conventional-

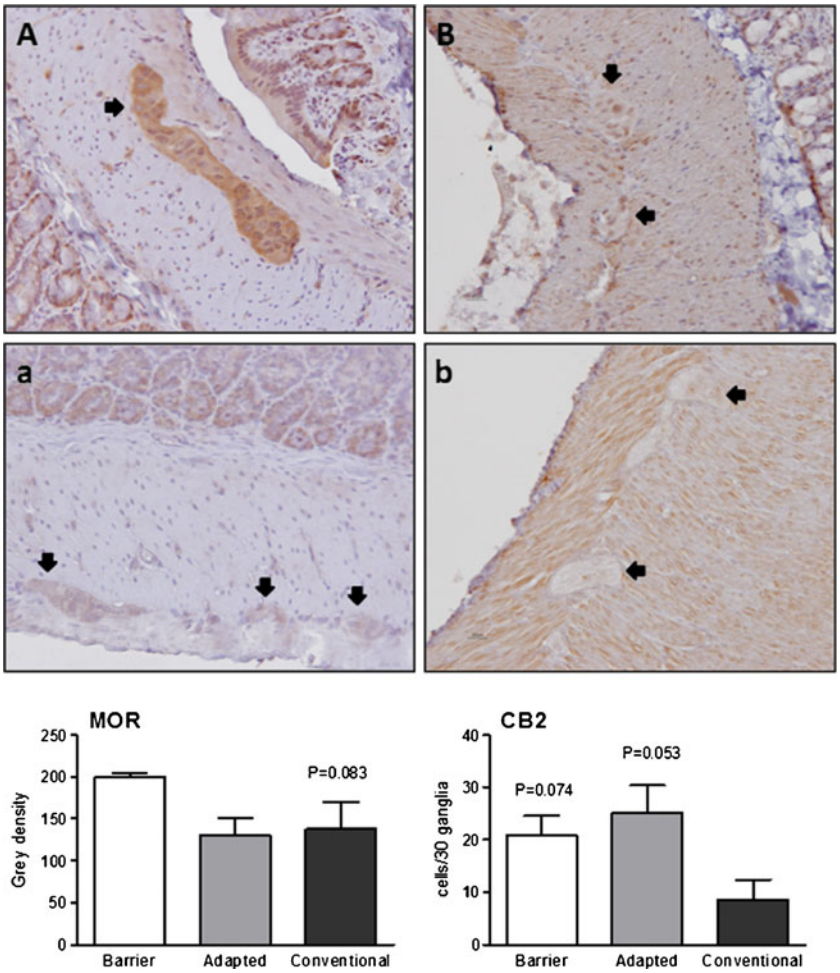


Figure 6 Expression of MOR and CB2 assessed by immunohistochemistry. Representative images (×200) showing the localization of MOR (**A**, **a**) and CB2 receptor (**B**, **b**) immunoreactivity in the myenteric plexus of the rat colon. Upper panels (**A**, **B**) correspond to a barrier-breed-and-maintained animal; lower panels (**a**, **b**) correspond to a conventional-breed-and-maintained animal. Arrows indicate myenteric ganglia. *Bar graphs* at the *bottom* show the quantification of MOR-like immunoreactivity in the myenteric ganglia and the number of CB2-immunoreactive cells within the myenteric plexus in the different experimental groups. Data are mean ± SEM (n=4 per group). Number on top of columns correspond to P values (ANOVA) vs. barrier for MOR and vs. conventional for CB2. *Barrier* barrier-breed-and-maintained group, *Adapted* barrier-breed adapted to conventional conditions group, *Conventional* conventional-breed-and-maintained group

breed-and-maintained animals compared with the barrier-breed-and-maintained group, in agreement with the mRNA expression results. This reinforces the view that these changes might translate into functional differences in CB2- and MOR-mediated responses.

As mentioned, the mechanisms through which GCM influences the expression of neuro-immune mediators remain largely unknown. Extensive work has demonstrated that the microbiota interacts with the internal milieu through specific bacterial recognition systems. These systems, with TLRs as main exponent, recognize bacterial components throughout specific epithelial receptors [8, 9, 20, 27, 28]. We previously showed that spontaneous adaptive variation of the gut microbiota is associated to minor changes in bacterial recognition systems, in particular TLR2 and TLR4 expression, and that the expression of these TLRs had no clear correlation with the microbiota [31]. The present results agree with these observations and show that spontaneous changes of GCM are not enough to directly modify the expression of TLR2, TLR4, TLR5, or TLR7. It can be speculated that profound changes in the microbiota, including the appearance of pathogenic strains, can be necessary to significantly alter host–microbe interactions, leading to changes in the expression of TLRs.

Overall, the present observations support the view that GCM is a dynamic system able to experience environmental-related adaptive changes. Moreover, data obtained suggest that the microbiota is able to interact with the host, leading to the modulation of endogenous regulatory systems. This might be part of the underlying mechanisms mediating the beneficial

effects of certain probiotics on gastrointestinal disorders. The present data directly implicate large spontaneous changes in gut commensal microbiota with the modulation of endogenous systems potentially implicated in sensory responses arising from the gut. These observations warrant further studies assessing how spontaneous or directed changes in gut commensal flora affect neural functions within the gut from a functional, morphological, and molecular point of view.

Acknowledgments We thank Emma Martínez and Antonio Acosta for their technical support in different stages of the study. This work was supported by grants BFU2009-08229 from the Spanish Ministerio de Ciencia e Innovación and 2009SGR-708 from the Generalitat de Catalunya. M. Aguilera received personal support from the FPI program (BES-2010-037699—Spanish Ministerio de Ciencia e Innovación).

References

1. Aguilera M, Vergara P, Martinez V (2013) Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. Neurogastroenterology & Motility, in press. doi:10.1111/nmo.12154
2. Amaral FA, Sachs D, Costa VV, Fagundes CT, Cisalpino D, Cunha TM, Ferreira SH, Cunha FQ, Silva TA, Nicoli JR, Vieira LQ, Souza DG, Teixeira MM (2008) Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 105:2193–2197
3. Blackshaw LA, Brierley SM, Hughes PA (2010) TRP channels: new targets for visceral pain. Gut 59:126–135
4. Brusberg M, Arvidsson S, Kang D, Larsson H, Lindstrom E, Martinez V (2009) CB1 receptors mediate the analgesic effects of cannabinoids on colorectal distension-induced visceral pain in rodents. J Neurosci 29:1554–1564
5. Bueno L (2008) Protease activated receptor 2: a new target for IBS treatment. Eur Rev Med Pharmacol Sci 12(Suppl 1):95–102
6. Camp JG, Kanther M, Semova I, Rawls JF (2009) Patterns and scales in gastrointestinal microbial ecology. Gastroenterology 136:1989–2002
7. Campbell JH, Foster CM, Vishnivetskaya T, Campbell AG, Yang ZK, Wymore A, Palumbo AV, Chesler EJ, Podar M (2012) Host genetic and environmental effects on mouse intestinal microbiota. ISME J 6:2033–2044
8. Cario E (2008) Therapeutic impact of toll-like receptors on inflammatory bowel diseases: a multiple-edged sword. Inflamm Bowel Dis 14:411–421
9. Carvalho FA, Aitken JD, Vijay-Kumar M, Gewirtz AT (2012) Toll-like receptor-gut microbiota interactions: perturb at your own risk! Annu Rev Physiol 74:177–198
10. Collins SM, Bercik P (2009) The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology 136:2003–2014
11. Coutts AA, Irving AJ, Mackie K, Pertwee RG, Anavi-Goffer S (2002) Localisation of cannabinoid CB(1) receptor immunoreactivity in the guinea pig and rat myenteric plexus. J Comp Neurol 448:410–422
12. Davis MP (2012) Drug management of visceral pain: concepts from basic research. Pain Res Treat 2012:265605
13. Dinoto A, Suksomcheep A, Ishizuka S, Kimura H, Hanada S, Kamagata Y, Asano K, Tomita F, Yokota A (2006) Modulation of rat cecal microbiota by administration of raffinose and encapsulated *Bifidobacterium breve*. Appl Environ Microbiol 72:784–792
14. Diop L, Guillou S, Durand H (2008) Probiotic food supplement reduces stress-induced gastrointestinal symptoms in volunteers: a double-blind, placebo-controlled, randomized trial. Nutr Res 28:1–5
15. Eutamene H, Lamine F, Chabo C, Theodorou V, Rochat F, Bergonzelli GE, Corthesy-Theulaz I, Fioramonti J, Bueno L (2007) Synergy between *Lactobacillus paracasei* and its bacterial products to counteract stress-induced gut permeability and sensitivity increase in rats. J Nutr 137:1901–1907
16. Fan YJ, Chen SJ, Yu YC, Si JM, Liu B (2006) A probiotic treatment containing *Lactobacillus*, *Bifidobacterium* and *Enterococcus* improves IBS symptoms in an open label trial. J Zhejiang Univ Sci B 7:987–991
17. Haarman M, Knol J (2006) Quantitative real-time PCR analysis of fecal *Lactobacillus* species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 72:2359–2365
18. Harmsen HJ, Raangs GC, He T, Degener JE, Welling GW (2002) Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl Environ Microbiol 68:2982–2990
19. Holzer P (2009) Opioid receptors in the gastrointestinal tract. Regul Pept 155:11–17
20. Honda K, Takeda K (2009) Regulatory mechanisms of immune responses to intestinal bacteria. Mucosal Immunol 2:187–196
21. Izzo AA, Sharkey KA (2010) Cannabinoids and the gut: new developments and emerging concepts. Pharmacol Ther 126:21–38
22. Kajander K, Hatakka K, Poussa T, Farkkila M, Korpela R (2005) A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. Aliment Pharmacol Ther 22:387–394
23. Kelly D, Mulder IE (2012) Microbiome and immunological interactions. Nutr Rev 70(Suppl 1):S18–S30
24. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25:402–408
25. Looijer-van Langen MA, Dieleman LA (2009) Prebiotics in chronic intestinal inflammation. Inflamm Bowel Dis 15:454–462
26. Ma BW, Bokulich NA, Castillo PA, Kananurak A, Underwood MA, Mills DA, Bevins CL (2012) Routine habitat change: a source of unrecognized transient alteration of intestinal microbiota in laboratory mice. PLoS One 7:e47416
27. Marques R, Boneca IG (2011) Expression and functional importance of innate immune receptors by intestinal epithelial cells. Cell Mol Life Sci 68:3661–3673
28. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118:229–241
29. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel JF, Ardid D, Desreumaux P (2007) *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 13:35–37
30. Selinummi J, Seppala J, Yli-Harja O, Puhakka JA (2005) Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 39:859–863
31. Teran-Ventura E, Roca M, Martin MT, Abarca ML, Martinez V, Vergara P (2010) Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats. Microb Ecol 60:691–702
32. Veerappan GR, Betteridge J, Young PE (2012) Probiotics for the treatment of inflammatory bowel disease. Curr Gastroenterol Rep 14:324–333
33. Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM (2006) Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 55:182–190
34. Verdu EF, Collins SM (2005) Irritable bowel syndrome and probiotics: from rationale to clinical use. Curr Opin Gastroenterol 21:697–701

35. Whelan K (2011) Probiotics and prebiotics in the management of irritable bowel syndrome: a review of recent clinical trials and systematic reviews. Curr Opin Clin Nutr Metab Care 14:581–587
36. Wildt S, Munck LK, Vinter-Jensen L, Hanse BF, Nordgaard-Lassen I, Christensen S, Avnstroem S, Rasmussen SN, Rumessen JJ (2006) Probiotic treatment of collagenous colitis: a randomized, double-blind, placebo-controlled trial with *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis*. Inflamm Bowel Dis 12:395–401
37. Williams EA, Stimpson J, Wang D, Plummer S, Garaiova I, Barker ME, Corfe BM (2009) Clinical trial: a multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study. Aliment Pharmacol Ther 29:97–103
38. Wright KL, Duncan M, Sharkey KA (2008) Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation. Br J Pharmacol 153:263–270
39. Xavier RJ, Podolsky DK (2007) Unravelling the pathogenesis of inflammatory bowel disease. Nature 448:427–434
40. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, Kitzweger E, Ruckser R, Haslberger AG (2011) Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PLoS One 6:e28654