Universitat Autònoma de Barcelona

STUDY OF THE ALTERATIONS IN THE INTESTINAL AND EXTRA-INTESTINAL MICROBIOME IN RATS AND HUMANS THROUGHOUT THE PROGRESSION OF CIRRHOSIS

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STUDY OF THE ALTERATIONS IN THE INTESTINAL AND EXTRA-INTESTINAL MICROBIOME IN RATS AND HUMANS THROUGHOUT THE PROGRESSION OF CIRRHOSIS

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In witness whereof, we hereby sign this document.

Barcelona, July 2018

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"The future belongs to those who believe in the beauty of their dreams"

Eleanor Roosevelt

LIST OF ABBREVIATIONS

16S rRNA, 16S ribosomal RNA

ACLF, acute-on-chronic liver failure

AF, ascitic fluid

AJs, adherens junctions

AMPs, antimicrobial peptides

APCs, antigen presenting cells

BT, bacterial translocation

CCL_4, carbon tetrachloride

CD, Crohn's disease

CDI, Clostridium difficile infection

CRC, colorectal cancer

CTLs, cytotoxic T lymphocytes

DCs, dendritic cells

ELISA, enzyme-linked immunosorbent assay

ENS, enteric nervous system

FDR, false discovery rate

FMT, faecal microbiota transplantation

GALT, gut-associated lymphoid tissue

GFP, green fluorescent protein

GI, gastrointestinal

HBV, hepatitis B virus

HCV, hepatitis C virus

IBD, inflammatory bowel disease

IBS, irritable bowel syndrome

ICC, ileo-cecal content

IECs, intestinal epithelial cells

IELs, intraepithelial lymphocytes

IFN-γ, interferon gamma

IL, interleukin

LBP, lipopolysaccharide binding protein

LPS, lipopolysaccharide

MLNs, mesenteric lymph nodes

MOD, multisystem organ dysfunction

MUC2, mucin-2

NO, nitric oxide

OTUs, operational taxonomic units

PAMPs, pathogen associated molecular patterns

PCoA, principal coordinate analysis

PCR, polymerase chain reaction

PHT, portal hypertension

PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PPs, Peyer's patches

PRRs, pattern recognition receptors

PSNS, parasympathetic nervous system

qPCR, quantitative PCR

rDNA, deoxyribonucleic acid recombinant

RES, reticuloendothelial system

RNS, reactive nitrogen species

ROS, reactive oxygen species

SBP, spontaneous bacterial peritonitis

SFB, segmented filamentous bacteria

SCFA, short chain fatty acids

SIBO, small intestinal bacterial overgrowth

sIGA, secretory immunoglobulin A

SIRS, systemic inflammatory response syndrome

SNS, sympathetic nervous system

TJs, tight junctions

TLRs, toll like receptors

TNF-α, tumour necrosis factor alpha

UC, ulcerative colitis

UPGM, unweighted pair group method with arithmetic mean

VLP, virus-like particles

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ANNEX 3. Supplementary material from Chapter 1.

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ANNEX 5. Publication related to the thesis. Sanchez *et al*. 2017 "Fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 reduces bacterial translocation in rats treated with carbon tetrachloride."

SUMMARY

The intestinal microbiota is known to play a pivotal role in the development of the host immune system and in the maintenance of host homeostasis by suppressing responses to pathogens and by enforcing the integrity of the barrier functions of the gut mucosa. Therefore, dysbiosis, described as the deviation in the composition and function of the gut microbiome, may affect the human health status and disease susceptibility. Different pathologies have been associated with changes in the composition of the intestinal microbiota, however, whether dysbiosis is a contributing factor or a consequence of the disease is still uncertain.

Cirrhosis is considered an important public health problem and is a progressive and chronic condition characterised by inflammation associated with dying hepatic cells and fibrosis, which lead to cellular dysfunction and portal hypertension. It is known that gut microbiota plays an important role in cirrhosis, bacterial translocation being a key factor in the pathogenesis and its complications.

Bacterial translocation is defined as the migration of viable bacteria or their products from the gastrointestinal tract to mesenteric lymph nodes and other extra-intestinal sites. Alterations of the normal intestinal microbiota, impairment of the immune status, and damage of the gut barrier that increases permeability, are the different mechanisms involved in translocation. It is hypothesised that alterations of microbial composition could favour bacterial translocation and activate an immune response with the consequent release of cytokines and an inflammatory response creating a feedback in which bacterial translocation promotes its own causative mechanisms.

Since bacterial translocation has not been extensively studied in humans and very little is known about the microbiome of extra-intestinal sites in patients with cirrhosis, we performed two studies in both humans and animals, to better understand the relevance of this phenomenon in liver cirrhosis. In the first study, we conclude that serum and ascitic fluid of patients with cirrhosis contain a complex and specific microbial community. Moreover, we propose that alteration of the serum and faecal microbiome composition could be considered indicators of cirrhosis progression. In the second study, we conclude that microbiome changes in intestinal sites are associated with microbial shifts in the mesenteric lymph nodes as well as

an increase in cytokine production, providing further evidence of the role of the gut-liver-immunity axis in the progression of cirrhosis.

RESUMEN

La microbiota intestinal desempeña un papel fundamental en el desarrollo del sistema inmune y en el mantenimiento de la homeostasis del huésped suprimiendo las respuestas a los patógenos y reforzando la integridad de las funciones de la barrera intestinal. Por lo tanto, una dysbiosis, descrita como una desviación en la composición y función, puede afectar al estado de salud y a la susceptibilidad a enfermedades. Diferentes patologías se han asociado con cambios en la composición de la microbiota intestinal, sin embargo, si la dysbiosis es un factor contribuyente o una consecuencia de la enfermedad todavía es incierto.

La cirrosis se considera un importante problema de salud pública, siendo una enfermedad progresiva y crónica caracterizada por una inflamación asociada con la muerte de las células hepáticas y fibrosis, conduciendo a una disfunción celular y a hipertensión portal. Se sabe que la microbiota intestinal desempeña un papel importante en la cirrosis, siendo la translocación bacteriana un factor clave en la patogénesis y sus complicaciones.

La translocación bacteriana se define como la migración de bacterias viables o sus productos del tracto gastrointestinal a los ganglios linfáticos mesentéricos y otros sitios extra-intestinales. Alteraciones en la microbiota intestinal normal, un deterioro del estado inmunitario y el daño en la barrera intestinal con el consecuente aumento en la permeabilidad, son los diferentes mecanismos implicados en esta translocación. Se hipotetiza que las alteraciones de la composición microbiana podrían favorecer la translocación bacteriana y activar una respuesta inmunitaria con la consiguiente liberación de citocinas y una respuesta inflamatoria que crea una retroalimentación en la que la translocación bacteriana promueve sus propios mecanismos causales.

Dado que la translocación bacteriana no se ha estudiado extensamente en humanos y se sabe muy poco sobre el microbioma de los sitios extra-intestinales, realizamos dos estudios en humanos y animales para comprender mejor la relevancia de este fenómeno en la cirrosis hepática. En el primer estudio, concluimos que la comunidad microbiana del suero y líquido ascítico es específico y complejo. Además, proponemos que la alteración de la composición microbiana en suero y heces podría considerarse un indicador de progresión de la cirrosis. En el segundo estudio, demostramos que los cambios del microbioma en el contenido intestinal se asocian con cambios microbianos en los ganglios linfáticos mesentéricos y un aumento en

la producción de citoquinas, proporcionando evidencia adicional del rol del eje hepato-intestinal e inmunidad en la progresión de la cirrosis.

INTRODUCTION

INTRODUCTION

1. GUT MICROBIOME

In the early 70's, the eco-system of microorganisms inhabiting our body was coined as the "human microbiota", which could add up to 100 trillion cells, tenfold the number of human cells. Moreover, the functions necessary to allow both an adequate growth of the microorganisms in the intestine as well as those required to maintain a good homeostasis of the entire ecosystem, are encoded by approximately 500,000 non-redundant genes per individual.[1–5]

The vast majority of these microorganisms belong to the gastrointestinal (GI) tract defining the "gut microbiota", previously called "indigenous flora".[2,6] This human intestinal microbiome is essential to the host's health, playing an important role in many aspects of the human physiology ranging from simple to complex processes.[4,5] In terms of metabolism it is able to generate energy and nutrients like amino acids and short chain fatty acids (SCFA) from substrates that are otherwise indigestible by the host such as undigested dietary carbohydrates (fibres).[7–10] Its role in pathogen resistance is characterised by a prevention of colonization through the production of antimicrobial peptides, as well as by the direct competition for attachment sites and nutrients.[9] The gut microbial community is also essential for the maturation and regulation of the immune system[11,12] and a normal gut development.[13] Lately, there has been a growing interest in the microbiota-gut-brain axis considering that it could be having an effect on brain chemistry and development, stress responses, behaviour and cognition.[14,15]

The gut microbiome comprises a complex colonisation of microorganisms in the GI tract,[3,16] including eukaryotes (mainly fungi), archaea, bacteria, and viruses. Within the gut microbiome, fungi, viruses and archaea are also considered to play a significant role in the host health. Greater access to a deeper sequencing technology has allowed a thorough study of this group of microorganisms.[17]

Fungi are considered to represent approximately 0.03% of the faecal microbiome, and despite the presence of mainly *Candida* and *Saccharomyces* having been found in several studies, there is no unanimity when defining a core mycobiome, perhaps due to its possible instability between individuals and in time.[17,18]

Viral communities are dominated by bacteria-infecting phage families, whereas eukaryotic viruses are found in lower abundance. A greater diversity has been observed at early ages, but their functional role has not yet been elucidated; being an increase of immunity or the protection against pathogens one of the possible functions. As a significant fact, it is worth mentioning a predominance of the virus-like particles (VLPs) belonging to the order Caudovirales of the phages in different studies.[17,19]

As for Archaea, the most remarkable characteristic would be that methanogens and Thermoplasmata are the only ones identified in human. Within methanogens the most found genus is *Methanobrevibacter* but further studies are needed to better understand and associate these microorganisms to human diseases.[20]

Although the intestinal microbiome, as has been shown, is much more complex, emphasis should be placed on bacteria, the most studied domain over a few decades since the use of only traditional culture methods. Around 10 million of non-redundant bacterial genes have been identified from 1200 individuals. They are encoded by about 1000 different bacterial species belonging to four phyla: Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria[21,22]; Firmicutes and Bacteroidetes groups make up 90% of the total bacteria (**Figure 1**).

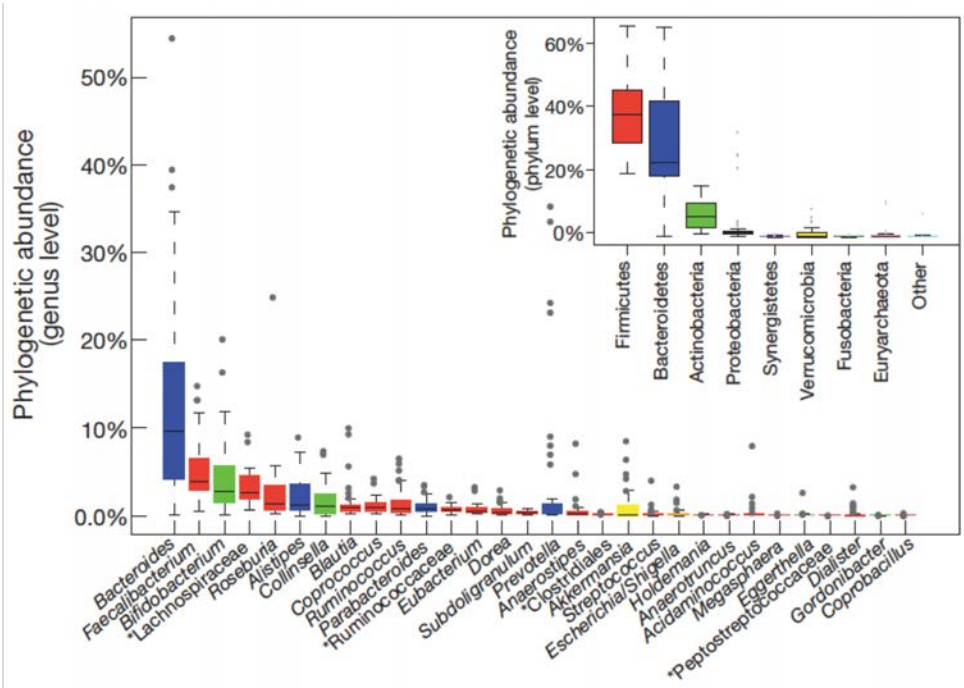


Figure 1. Bacterial abundances at the genus and phylum level in the human gut. The figure is a copy from Arumugam *et al*., Nature, 2011.

The composition of the intestinal microbes varies among individuals, and also between intestinal sites in the same individual. Microbial diversity along the GI tract, which refers to the oral cavity till the rectum passing through the oesophagus, stomach and intestines, depends on the physiological properties that are shaping each section including chemical (presence of different levels of acid, oxygen availability, tissue structure and transit time) and nutritional gradients, as well as the immune system activity.[4,5,16,21,23–28]

1.1. CORE GUT MICROBIOME

Over the last decade, due to the development of the Next Generation High Throughput Sequencing Techniques, there has been significant progress in defining the role of the gut microbiome in health and disease. Several studies have focused on the characterization of a common core microbiome, even though it consists predominantly of bacteria. Defining a core microbiome is challenging, as the complexity is quite high, owing to the variability existing within the same individual over time, and in between individuals due to their genetic background, host physiology and environmental exposure among other factors.[29,30] Using shotgun sequencing and a 90% identity threshold, Qin *et al.* found that 18 species were identified to be common in all individuals and 57 species in 90% individuals at a 1% coverage. Additionally, further investigation revealed a high inter-individual variability (about more than an order of magnitude) in terms of abundance frequency while the abundance of metabolic pathways was more consistent.[4,31]

It has been hypothesised for years that the gut core microbiota referred to the number of species that are common to the gut of all humans. However, it is now possible that what could exist, is a gene-level core microbiome where different combinations of species could fulfil the same functional roles within a particular habitat. Several studies revealed the presence of common functional gene groups encoding for housekeeping functions, processes specific to human-associated microbiome across body-site habitats and specialised core functions for each habitat as a part of the so called core microbiome.[29–33]

Indeed, the large number of factors that influence microbial community structure such as host genetics, environment, lifestyle, age or drug administration throughout life make its analysis more complex.[34] In fact, as described by Dominguez-Bello *et al*., already early in life there are factors that modulate the microbial community such as the delivery method. Natural birth

favours the presence of vagina-dominating bacteria while C-section delivery leads to a dominance of skin-harbouring bacteria.[35] Furthermore, several studies have reported that infant feeding is another important modulating factor of the intestinal bacterial colonization entailing some differences between breast milk-fed and formula-fed infants.[36,37] But it is not until the age of 3 years when the intestinal microbiome acquires similar characteristics to that of adults in both diversity and functionality and reaches a relative stability over time.[37,38]

Moreover, the size and nature of the selected cohort, the methodological aspects such as the extraction method or the choice of the hypervariable region of the 16S ribosomal RNA gene (16S rRNA), the depth of the analysis and the bioinformatic approaches among others also influence the outcome of studies about the core microbiome.[39,40]

1.2. DYSBIOSIS

Greater richness and diversity of microbial species in the human intestine may be an indicator of health. On the contrary, dysbiosis, described as a deviation in the composition and function of the intestinal microbiota, is typically related to a loss of diversity and low-grade spontaneous inflammation at the mucosal barrier (**Figure 2**).[41,42] Different factors including diet, drugs, intestinal mucosa as well as genetics, health status, immune system, lifestyle and environment of the human host contribute to moderate shifts in the microbial composition. But other aggravating factors like oxidative stress, presence of bacteriophages and bacteriocins are those that could cause an exacerbation of the initial status to the point of dysbiosis.[43–46]

The functional implications of dysbiosis are still poorly understood, but may affect human physiology, health status and disease susceptibility. It is already known that different pathologies are associated with changes in the composition of the intestinal microbiota; however, whether dysbiosis is a contributing factor or a consequence of the disease is far to ascertain. [47,48]

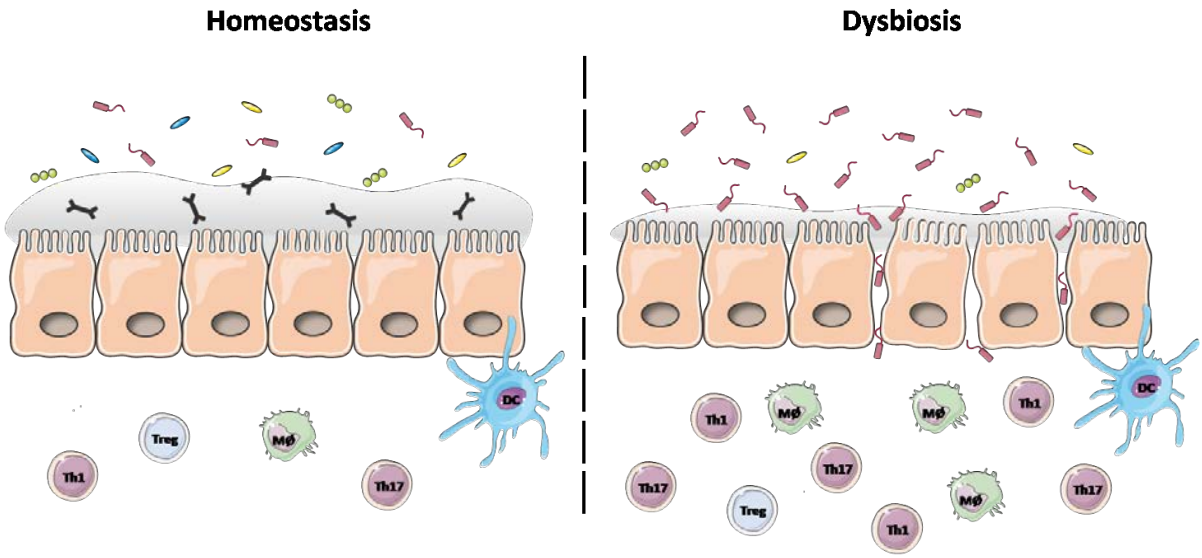


Figure 2. Schematic representation of the GI tract under normal and dysbiotic conditions. Dysbiosis is represented by a change in the microbial community. Moreover, a disruption of the mucus layer and an activation of certain immune cells (Th1, Th17 and macrophages) is also shown. Treg = regulatory T cells; Th1 and Th17 = helper T cells; MØ = macrophages; DC = dendritic cells.

Several studies have demonstrated that a disruption of the gut microbiota is linked not only to various gastrointestinal disorders, but also to systemic diseases due to the important role of the microbiota in maintaining host homeostasis. Intestinal disorders include Irritable Bowel Syndrome (IBS), Inflammatory Bowel Disease (IBD), coeliac disease, *Clostridium difficile* infection (CDI) and colorectal cancer (CRC) while extra-intestinal disorders include metabolic disorders, autoimmune diseases and neurological disorders.[28,41,46,47,49]

A microbial imbalance may explain a large part in the clinical manifestations of functional gastrointestinal disorders even though we still do not have conclusive scientific evidence.[50] Several studies have described differences in microbial composition in the different subtypes of **IBS** with respect to asymptomatic patients. However, the results are inconsistent between the different studies, which may be due to the lack of the use of standardised sample processing methods and partly due to the heterogeneous nature of the disease.[47,51–53] For instance, Pozuelo *et al*. reported that there were not significant differences between healthy controls and IBS patients with constipation. This finding differed to previous results in which this subtype presented a higher abundance of *Veillonella* and *Ruminococcus*.[54]

Although the aetiology of **IBD** is unknown, there is also increasing evidence that dysbiosis has a role in its pathogenesis, but it is still unresolved if tissue damage results from an abnormal immune response to a normal microbiota or from a normal immune response against an abnormal microbiota.[5,55–59] Within IBD, Crohn's disease (CD) and ulcerative colitis (UC) are the most prevalent forms, the former being the one with a greater dysbiosis characterised by a lower microbial diversity, a more altered microbiome composition and a more unstable microbial community.[60]

Recently, changes in microbial composition have also been described in **coeliac disease**, both in faecal samples and intestinal biopsies, characterised by an increase in Gram-negative bacteria such as *Bacteroides* and a reduction in the number of Gram-positive bacteria like *Bifidobacterium*. Thereby, changes in the composition of the intestinal microbiota along with an alteration of the epithelial barrier could precede a specific gluten-dependent immune response.[47,61,62]

Among one of the multidrug-resistant microorganisms related disease is CDI, in which an antibiotic treatment can lead to perpetuate its recurrence favouring the appearance of recurrent CDI and the impossibility then to treat with any antibiotic.[63] In addition, it has been described that other factors could favour the appearance of this infection by also causing an imbalance in commensal microorganisms facilitating the presence of *C. difficile* such as H2-receptor antagonists and proton-pump inhibitors by the suppression of gastric acid or the prolonged use of specific diets in critically ill patients that could deprive the colonic microbiota of their source of nutrients.[64]

Over the last decade, there has been a growing interest in investigating the involvement of the microbiota in the pathophysiology of **CRC**. It appears that a complex interrelation between diet, gut microbiota and CRC could exist. Thus, it has been shown that a dysbiosis could be linked to CRC through bacteria-derived metabolites produced by the fermentation of dietary ingredients. Possible toxic and genotoxic metabolites show a tendency to bind specific intestinal cell surface receptors affecting intracellular signal transduction.[47,65,66]

Changes in the abundance of certain phyla have been associated with metabolic syndrome. It has been suggested that a decrease in Bacteroidetes and an increase in Firmicutes could be a differential footprint for the initiation and/or progression of these diseases.[65,67,68] Several pathways including energy harvest, modulation of plasma glucose and serum lipids and low-

grade intestinal inflammation may contribute to the development of insulin resistance, diabetes and obesity.[47,68]

Moreover, other extra-intestinal diseases have also been associated with dysbiosis like autoimmune diseases[69] or those related to the "gut brain axis" affecting behaviour and cognitive function.[47]

As described, different pathologies have been associated with changes in the composition of the intestinal microbiota, however, it is still unclear whether this association implies causality, or it is a consequence of a given disease. To establish an aetiological role, more interventional studies investigating the maintenance, improvement or restoration of the microbiota are required to go in depth and to better understand the mechanisms involved. One of the ideal scenarios to accomplish this purpose is the use of "humanised" gnotobiotic animal models.[41,47] In the near future, the microbiome replacement procedures based upon faecal microbiota transplantation (FMT) or cocktail microbial transplantation may be a real and effective option as a single or adjuvant as alternatives to conventional treatments for a set of diseases.[70]

FMT is the transfer of intestinal microbiota through a faecal suspension from a healthy donor to a patient so as to normalise an altered gut microbiota in terms of composition and functionality.[63] So far, FMT has only been shown to be clearly effective as a rescue treatment for recurrent cases of CDI when standard therapy fails.[71] However, although it is gaining interest in other pathologies, no significant results have been seen until now.[63] This is the case of IBD, where an attempt has been made to elucidate the efficacy of this type of treatment, mainly in UC, obtaining unclear results. Moreover, there are still limitations in the process due to the lack of unanimity regarding the screening and selection of the donor, the preparation and administration of the suspension, and mostly concerning the lack of information about the safety profile.[72,73]

2. INTESTINAL EPITHELIAL BARRIER

The epithelium lining the GI tract makes up the largest interface between the luminal content and the external environment of the host, having a key role in preserving the milieu intérieur.[74,75] The intestinal barrier is a complex structure that needs to function as a selective barrier allowing the absorption of nutrients, water and electrolytes, but simultaneously being

a defensive barrier limiting the passage of harmful molecules such as toxins or microorganisms. An appropriate regulation of these functions through an interplay between physical, cellular and chemical components is needed in order to ensure inner homeostasis and maintenance of the mucosal barrier.[76,77]

The intestinal mucosa is built up by several elements which can be categorised as extracellular and cellular components according to their nature and anatomical location (**Figure 3**).[78]

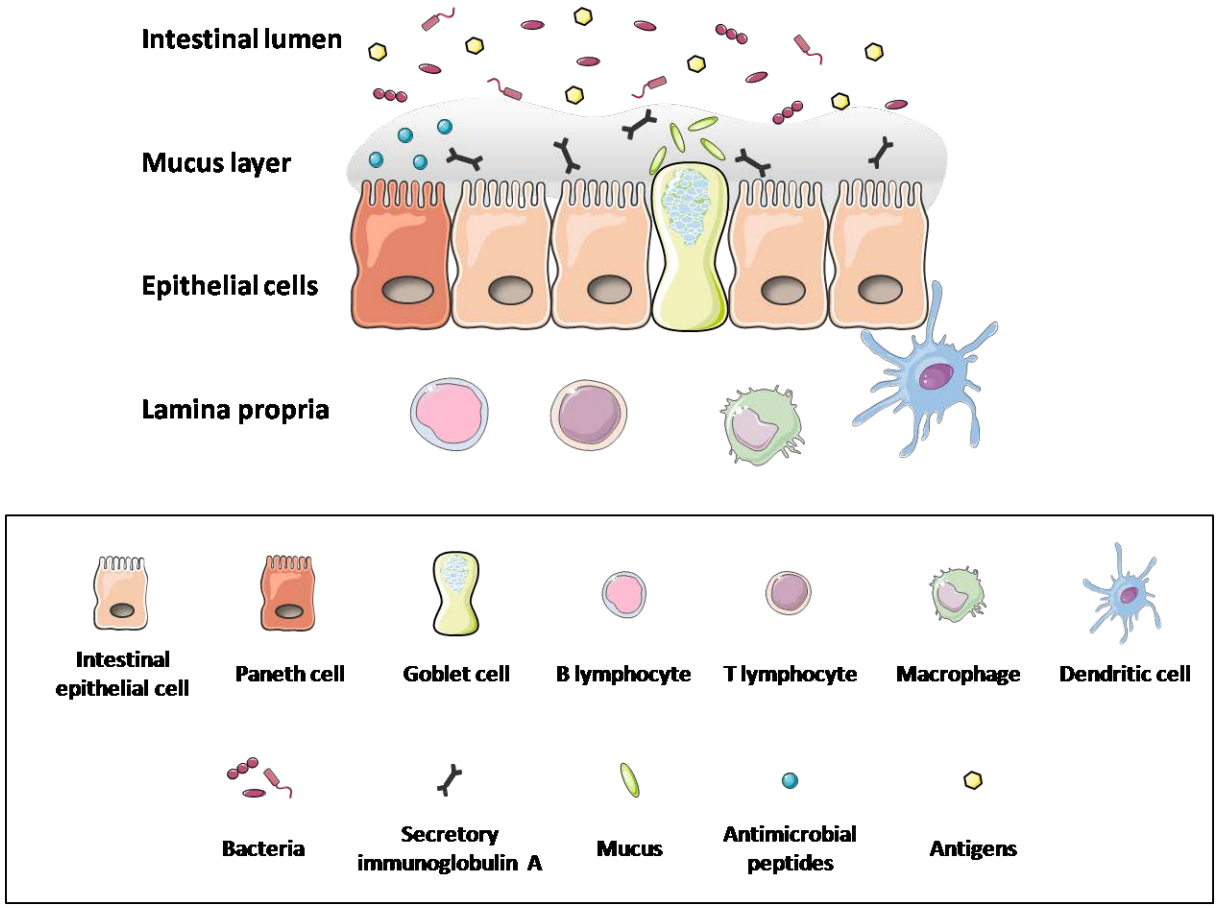


Figure 3. Characterisation of the intestinal epithelial barrier. Representation of the main components of the intestinal barrier which includes a mucus layer; a single layer composed mainly of enterocytes which also includes Paneth cells, goblet cells, and microfold cells and enteroendocrine cells (the latter are not represented in the present figure); and the lamina propria composed of loose connective tissue which includes the gut-associated lymphoid tissue.

2.1. INTESTINAL EPITHELIAL BARRIER ANATOMY

2.1.1. MUCUS LAYER

The mucus layer is considered as the first line of physical defence. While in the small intestine the mucus layer is thinner and not well defined allowing the absorption of nutrients, the contrary occurs in the colon.[79,80] The main component of this layer is mucin, more precisely mucin-2 (MUC2) secreted by goblet cells in the epithelial layer, making up a gel-like structure. Moreover, it contains commensal gut microbiota, secretory immunoglobulin A (sIGA) and antimicrobial peptides (AMPs) secreted by Paneth cells that help to limit direct contact between bacteria and intestinal epithelial cells (IECs).[76,81] In the colon, within the mucus membrane, two layers can be well distinguished: an outer, loose layer, where bacteria reside, and a normally sterile inner dense layer.[77,79]

2.1.2. EPITHELIAL LAYER

Underneath the mucus layer an epithelial layer is settled. It is a single, continuous and polarised layer that separates the lumen from the lamina propria. The different and specialised IECs forming this layer emerge from pluripotent stem cells residing at the crypts that differentiate during the migration towards the villi.[81,82] Although most of the cells found in the monolayer are enterocytes, constituting up to 80%, they co-exist with other cell types like microfold cells (M cells), goblet cells, Paneth cells and enteroendocrine cells.[83,84] Moreover, intraepithelial lymphocytes (IELs) are found dispersed between the adjacent epithelial cells.[85]

Interaction between IECs through a protein network that links adjacent cells and seals the intercellular space is important for maintaining intestinal barrier integrity.[75] The protein connections between adjacent cells are composed by important complexes: tight junctions (TJs), adherens junctions (AJs), desmosomes and gap junctions (**Figure 4**).[61] These complexes can be classified into three functional groups including tight junctions, anchoring junctions and communicating junctions.

- **Tight junctions** reside in the apical section of enterocytes and they act as a gate in the paracellular transport controlling the entrance of ions, solutes and luminal antigens. Tight junctions are composed of transmembrane proteins including occludin, claudins,

junctional adhesion molecules (JAM) and tricellulin which are linked to cytoskeletal actomyosin fibres by scaffold proteins represented by zonula occludens.[86]

- **Anchoring junctions** include the AJs found below the TJs and composed of cadherines, and the desmosomes mainly comprised of desmoglein, desmocollin and desmoplakin. Their function is to maintain, through strong adhesive bonds, the physical connection of adjacent cells.[76,87,88]

- **Communicating junctions** or gap junctions are comprised of connexins that allow the exchange of ions and small molecules of neighbouring cells by setting up a channel through their membranes. Connexins are also thought to play a crucial role in development, cell growth and cell differentiation.[89]

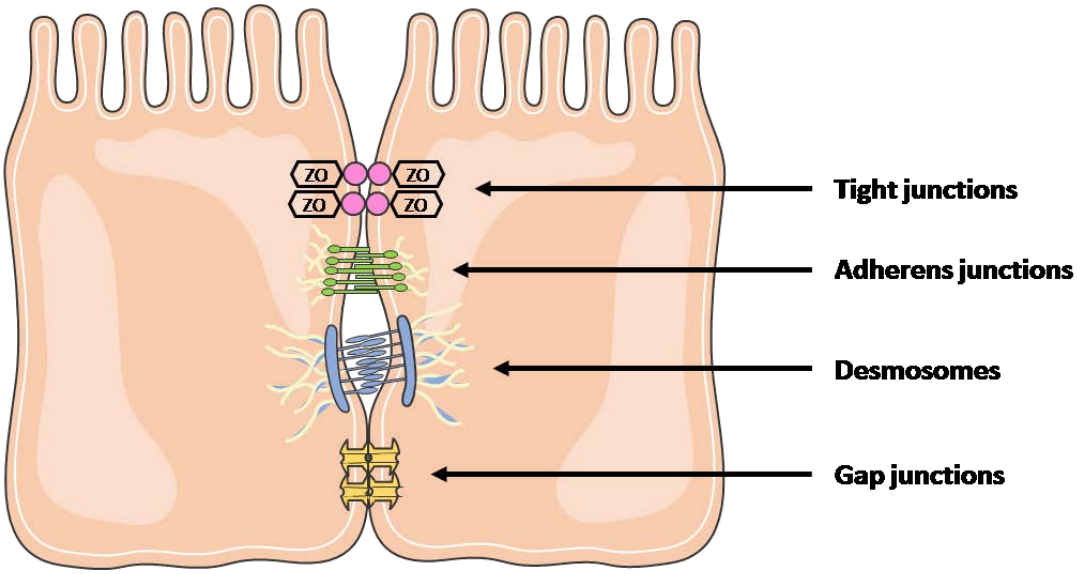


Figure 4. Main protein connections between adjacent cells. Schematic representation of the three main functional groups including tight junctions, anchoring junctions which comprise adherens junctions and desmosomes, and gap junctions.

2.1.3. LAMINA PROPRIA

The lamina propria is located beneath the epithelial layer, is composed of a loose connective tissue[80] and harbours immune cells (including macrophages, dendritic cells (DCs), plasma cells and lymphocytes), the enteric nervous system and connective tissue.[84,90] The gut-associated lymphoid tissue (GALT), considered as the largest immune organ in the body, is made up of these gastrointestinal tract immune cells and can be divided anatomically in two compartments:[91]

- **Organised GALT**, induces immune responses, and consists of lymphoid follicles isolated along the intestine, associated lymphoid follicles or Peyer's patches (PPs) and mesenteric lymph nodes (MLNs).[91] PPs are composed of several B cell follicles separated by areas containing T cells, and macrophages and dendritic cells (DCs), having both an important role as antigen presenting cells (APCs) in the GALT.[80,92] MLNs are the largest lymph nodes in the body and they also comprise a large number of lymphocytes and APCs. They are recognised for being the filter of the lymph that comes from the intestine and they act accordingly against incoming antigens.[93]

- **Diffuse GALT,** the effector of immune responses, integrates populations of scattered lymphocytes such as IELs or lamina propria lymphocytes as well as plasma cells, specially isotype IgA, and a small proportion of B cells, macrophages and DCs.[91,94] Effector T cells such as helper T cells (Th1, Th2 and Th17) derive from a pool of naïve CD4+ T lymphocytes. Each lineage is characterised by the secretion of specific cytokines like IFN-γ, IL-4 and IL-17, among others, respectively. These cells coexist with regulatory T cells (Treg) which suppress immune responses to maintain gut homeostasis[95–97] and CD8+ cytotoxic T lymphocytes (CTLs) which can kill infected cells.[80]

Although the enteric nervous system (ENS) can function autonomously, it is connected to the central nervous system through parasympathetic (PSNS) and sympathetic nervous systems (SNS). These systems regulate important GI functions such as digestion and motility.[98,99] Besides its known functions, the SNS has also been seen that it would be associated with the mucosa and the GALT. Indeed, sympathetic neurotransmitters could modulate the response of both innate and adaptive immunity. For instance, norepinephrine is able to increase

proinflammatory response through activation of β adrenergic receptors or have a dual role in the innate immunity activating specific receptors depending on the concentration of the neurotransmitter.[100]

2.2. ANTIGEN SAMPLING AND RECOGNITION

The intestinal mucosa is in constant interaction with commensal microbiota as well as with a variety of pathogens. In addition, since this is the first antigen entry site, the immune system must be able to discern between commensal and potentially harmful bacteria to trigger an appropriate immune response. So, a good monitoring of the luminal microenvironment to the immune system is needed.[101,102]

Several pathways related to entry of antigens and presentation from the intestinal lumen to the immune system have been described.[80,103]

a. **Passage through the M cells**: for years it has been postulated that one of the most important functions of the M cells was to act as one of the main entry routes of antigens up to the underlying mucosal immune system, specially DCs through pinocytosis and receptor-mediated endocytosis.[103,104] Thus, antigens can be presented directly from DCs to T cells or via MLNs.[80]

b. **Transepithelial dendrites**: it is a direct route in which the dendrites from DCs penetrate the epithelium through tight junctions and sample the antigen from the intestinal lumen. However the projection of these dendrites seems to be restricted to specific DCs that express the chemokine receptor CX3CR1.[92,103]

c. **Goblet-cell-Associated Antigen Passages**: this route is the most recently studied. As described by McDole *et al*., it implies the passage of small molecular weight soluble antigens through the goblet cells located in the small intestine to the specific CD103+ DCs.[105]

d. **Passage through enterocytes**: the monolayer of enterocytes that form the intestinal epithelium is a selective permeable barrier that allows the passage of specific

molecules. The entrance of these solutes is regulated by two main routes that will be discussed extensively in the next section.[103]

2.3. INTESTINAL PERMEABILITY

The intestinal barrier should not be seen as a static, but as a dynamic and responsive structure to both internal and exogenous stimuli.[78] When talking about intestinal permeability in this context, we refer to the selective ability of the intestinal barrier to prevent the passage of harmful substances from the external environment while permitting the passage of desired smaller molecules.[106] Thus, the entrance of solutes and molecules is dependent on a selective process in which two major pathways can be involved:

- The **paracellular route** is regulated by tight junctions and allows the passage of molecules between two adjacent epithelial cells. It is known that there are two different pathways within this route.[78] On one hand, there is the so-called "leak pathway" that allows the passage of large solutes except whole bacteria. On the other hand exists the "pore pathway" that is characterised by small pores and allows the passage of molecules with a radius less than 4 Å.[78]

- The **transcellular route** allows solute transportation across the membrane of the enterocyte. This pathway comprises the passage of substances via passive diffusion, active carrier-mediated transport or endocytosis which can be divided into two categories, phagocytosis or "cell eating" (the uptake of large particles) and pinocytosis or "cell drinking" (the uptake of fluid and solutes).[107,108]

Thus, an increase of the epithelial barrier permeability and a decreased mucosal defence may lead to enhance translocation of luminal content and subsequent activation of the intestinal mucosal immune system, which could contribute to the development of a disease condition.

3. BACTERIAL TRANSLOCATION

In 1979, Berg *et al*. introduced the term bacterial translocation (BT) defining it as the passage of viable bacteria from the gastrointestinal tract through the epithelial mucosa into the lamina

propria and then to the mesenteric lymph nodes and possibly other organs.[109] Thanks to the advances in molecular biology, the later studies showed that not only viable bacteria were involved in translocation but also other bacterial compounds such as endotoxins, antigens or bacterial DNA were able to cross the intestinal epithelial barrier and reach extra-intestinal sites.[110–112]

In the 80s, there was an increased interest in demonstrating which bacteria were able to translocate more frequently.[113] Later studies confirmed that gram-negative bacteria had a great rate of translocation, specially facultative anaerobic Enterobacteriaceae, such as *Escherichia coli*, *Klebsiella pneumoniae* or *Proteus mirabilis*.[110,114] Besides, Ljungdahl *et al*. showed that some strains, due to a better adherence and ability to attach to the mucus, had a greater capacity for translocation.[115]

Nowadays, thanks to metaomic approaches, it has been demonstrated that not only bacteria can translocate, but also fungi.[116] Thus, despite the subject of the present thesis being "bacterial translocation" we should keep an open approach to "microbial translocation".

3.1. MECHANISMS OF BACTERIAL TRANSLOCATION

Disruption of the ecological balance of the normal intestinal microbiota, resulting in small bowel bacterial overgrowth, states of systemic immuno-suppression, and damage of the gut barrier that increases permeability, are the different mechanisms postulated to be responsible for bacterial translocation previously described by Berg *et al*. (**Figure 5**).[110,112,117]

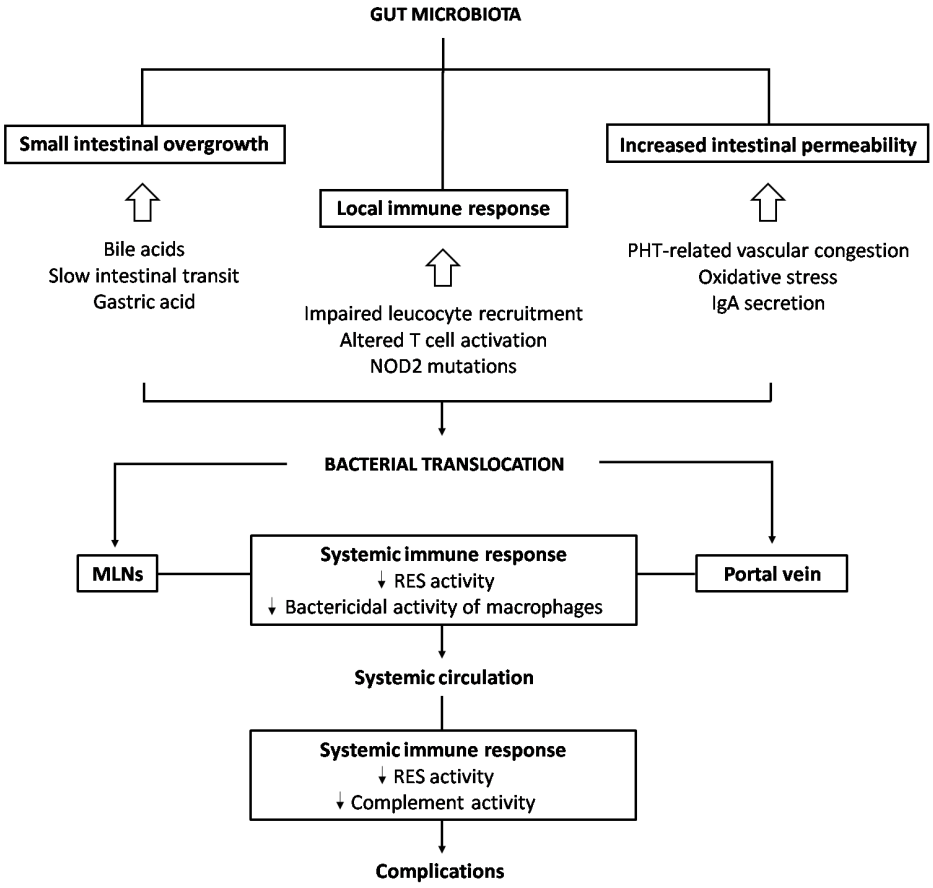


Figure 5. The different mechanisms involved in bacterial translocation. The three main mechanisms that favour the appearance of bacterial translocation. This figure is modified from Bellot *et al*., Liver International, 2012.

3.1.1. SMALL INTESTINAL BACTERIAL OVERGROWTH (SIBO)

As previously described, the intestinal microbiota plays an important role in physiological functions, constitutes an ecological barrier for the microorganisms by producing antimicrobial compounds and thus provides resistance against colonization.[5,112] Besides, it has been demonstrated in studies with germ-free animals the importance of the commensal microbiota in the development and maturation of the intestinal immune system.[81,118]

Therefore, an alteration of the gut microbiota may constitute a risk factor in the development of BT. Indeed, a small intestinal overgrowth defined as an increase in the number and/or an alteration of the type of bacteria present in the small bowel has been assumed as one of the main factors promoting BT.[117] Actually, Steffen *et al*. demonstrated that the number of viable bacteria of a certain strain in the MLNs was directly correlated with their levels in the

caecum.[119] In line with these findings, another study showed that almost all the bacteria translocated to MLNs were also overgrowing in the stool.[120]

Reduced gastric acidity, slowed gastrointestinal motility, intestinal immunological factors and pancreatic and biliary secretions are the factors that could favour the appearance of SIBO.[117,121,122]

3.1.2. INCREASED INTESTINAL PERMEABILITY

Diverse mechanisms exist to control the adhesion of bacteria to the epithelium. They include secretion of mucin by goblet cells (3 L/day), secretion of gastric acid and bile, of pancreatic enzymes or the production of IgA contained in the mucosal surfaces that could inhibit bacterial overgrowth. Even though those mechanisms are considered of great importance prior to the epithelial penetration, the defence mechanism that plays a more critical role is the epithelium in essence.[117,123]

As previously mentioned, the entrance of luminal antigens through the epithelium can be carried out by two different routes, the paracellular and the transcellular. Several years ago, Alexander *et al*.[124] already described that the transcellular pathway was the major route of choice for bacterial translocation even though the enterocytes were morphologically intact.[112,125] Notwithstanding, most studies done on intestinal permeability were based on the paracellular route.[117]

Intestinal permeability is understood as a functional feature of the intestinal epithelial barrier, and it refers to the passage of molecules across this barrier.[126] It has been shown that the passage of whole bacteria between IECs through TJs is not possible due to its size. Therefore, an impairment in the "leak pathway" with an increase in the permeability at TJs complex is hypothesised to allow bacterial entrance, resulting in the activation of inflammatory responses.[127]

The factors that could be involved in the increase of the epithelial barrier's permeability, as previously summarised in **Figure 5**, would be the dilatation of intercellular spaces in the intestinal epithelium, submucosal inflammatory changes and vascular congestion.[117]

Oxidative stress and appearance of reactive oxygen species (ROS) causing structural and functional damage to the intestine may be involved in an increase of the intestinal permeability.[128] In normal conditions, excessive production of ROS, which is essential at low levels for different processes of the cell such as regulatory mechanisms, intracellular signalling or host defence against invading microbes,[129] is compensated with an antioxidant defence system that comprises antioxidant enzymes and vitamins. In inflammatory conditions, an increased release of ROS by phagocytic cells as a defence mechanism leads to oxidative stress. So the activation of immune cells entails the overproduction of harmful substances which could produce a negative feedback increasing the inflammation and consequently causing epithelial damage.[130–132] Similarly, reactive nitrogen species (RNS) such as nitric oxide (NO) have a comparable behaviour to ROS, generating a nitrosative stress that produces an epithelial barrier dysfunction.[133,134]

In addition, it is worth mentioning the regulatory role of NO on the vascular tone due to its vasodilator effect.[135] It has been described that a disturbance of the hepatic vascular structure leads to an increase in the resistance to portal blood which along with an increase in the blood flow due to the splanchnic vasodilation favoured by the production of NO results in portal hypertension (PHT).[136,137] This vasodilation leads to the onset of hyperdynamic circulatory state which implies the stimulation of compensatory vasoconstrictor mechanisms that result in sodium retention responsible for the appearance of ascites.[138] Moreover, there is a decrease in the mucosal perfusion due to a vascular congestion and intestinal oedema formation allowing the correlation between PHT and an increase in intestinal permeability.[139]

Moreover, the integrity of the intestinal mucosa could also be affected by endotoxemia, as it was described some years ago by O'Dwyer *et al*.[140] and possibly due to the release of specific cytokines[141], or hypoxia and acidosis. It is known that cell damage is likely to occur when there is a decrease in tissue oxygenation (hypoxia). In addition, it has been a direct relationship between the existence of ischemia, the presence of acidosis and the degree of intestinal permeability. Actually, acidosis could favour the production of ROS and therefore hyperpermeability.[123,142]

Even so, increased intestinal permeability cannot be considered by itself as a decisive factor explaining the pathophysiology of BT.[117]

3.1.3. IMPAIRMENT OF THE IMMUNE STATUS

The immunological barrier, constituted by the GALT, is indispensable to offer protection at a local level and as a consequence, to prevent systemic inflammation and dissemination. As already demonstrated in a study carried out by Berg *et al*., immunosuppression caused by a series of immunosuppressive agents could increase bacterial translocation ratio.[143]

Indeed, bacteria that translocate are phagocyted and neutralised to avoid their growth and dissemination. So the dysfunction of some component of the GALT system may favour the appearance of BT and the development of a systemic immune response.[117,144]

The importance of intestinal T lymphocytes to confer protection against bacterial translocation was suggested in the 80s and confirmed years later by Gautreaux *et al*. achieving a decrease in the translocation of *E. coli* through adoptively transferred T cells to mice previously depleted of these cells.[145] Furthermore, the suppression of intestinal T cells could favour the accumulation of bacteria in MLNs and its spreading to extra-intestinal sites.[146] Hence, an altered T cell activation may be affecting the maintenance of an appropriate immune system status against bacterial translocation.

Some alterations of the local immune system could be a consequence of genetic alterations as shown by several studies. For instance, the risk of appearance of spontaneous bacterial peritonitis (SBP) is increased by variations in NOD2 protein.[147,148] NOD2 belongs to the so-called pattern recognition receptors (PRRs) which play an important role in the innate immune system recognizing microbes through conserved molecular structures called pathogen associated molecular patterns (PAMPs).[149] So, an alteration of these receptors could influence the host immune response by altering the detection and removal of microbial pathogens.

Moreover, it should be noted that leukocyte recruitment accomplished by diverse adhesion molecules such as selectins is needed as an early step for an effective defence against pathogens. Thus, an impairment in this mechanism would inhibit the inflammatory response.[150,151]

It should be specified that after overcoming the epithelial barrier and reaching the lamina propria, there are various mechanisms of systemic dissemination of the bacteria:[112]

- **Lymphatic enteric drainage** is considered the main route of translocation, with the MLN as the first or only point where viable commensal, pathogenic bacteria or other PAMPs can be detected.

- **Portal venous system** with the liver as the first receptor organ. It is not considered as a critical way in BT since in several studies no correlation was demonstrated between the presence of bacteria and endotoxins in portal vein and systemic inflammatory response.

Once bacteria have managed to translocate, phagocytosis and neutralization are parts of the last mechanisms of the host defence to prevent their growth and consequent bacteraemia or other infections.[117] Thus, a reduction of the reticuloendothelial system (RES) activity, which is made up of phagocytic cells like Kupffer cells in the liver, may explain the increase of bacteria and endotoxins in systemic circulation.[152]

In any case, microbial translocation entails a chronic inflammatory response with a release of proinflammatory cytokines (interleukin (IL)-6 and -12), tumour necrosis factor-alpha (TNF)-α, interferon-gamma (IFN)-γ) and other effector molecules such as NO causing a worsening of the integrity of the intestinal barrier and thus leading to a vicious circle perpetuating the mechanisms that cause BT.[117,153]

Even so, microbial translocation is not always synonymous of disease, it is a process that can also occur under normal physiological conditions without causing damage.[112]

3.2 BACTERIAL TRANSLOCATION AS A PHYSIOLOGICAL PROCESS

It has been known for years that BT can be a physiological process occurring in healthy individuals as well as in animals.[143,154] With the appearance of the culture independent molecular techniques based on 16S rDNA gene sequencing, the presence of DNA was confirmed in MLNs of control rats suggesting that BT is a physiological feature and should be considered as a constant and polymicrobial phenomenon.[155]

Furthermore, it has been stated that the baseline rate of translocation in human studies is 5% to 10% while the normal rate in animals is slightly higher, ranging approximately between 10% to 20%.[114]

Bacterial translocation could even be considered as an essential biological phenomenon acting as a regulator of tolerance, and local and systemic immune responses. The capture of the different antigens that are in contact with the intestinal epithelium could be used for the benefit of the host for the production of immunocompetent cells.[114,156]

In addition, and as a clear example of translocation without deleterious consequences for the host, it has been shown that some commensal alive bacteria as well as bacterial DNA coming from the gastrointestinal tract could pass to the mammary glands and be found in breast milk. A study performed by Perez *et al*. in mice demonstrated that physiological BT could increase during pregnancy and lactation period.[157]

3.3 BACTERIAL TRANSLOCATION IN DISEASE

BT goes from being a physiological phenomenon to a clinically significant one when infectious complications appear and there is an increase in the risk of morbidity and mortality in patients. Diverse studies have demonstrated that BT is quite frequent, occurring in 4-59% of the patients depending on the basal clinical condition. Interestingly, although the presence of BT is linked to a higher frequency of septic complications, most patients with evidence of translocation lack infectious complications.[112]

BT has been associated with numerous serious pathologies such as: haemorrhagic shock, acute pancreatitis, obstructive jaundice, abdominal surgery, intestinal obstruction, liver cirrhosis, large burns, severe trauma and polytrauma, aortic aneurysm repair, cardiovascular bypass, heart failure, and malignancy.[158] The clinical consequences that implies BT in these conditions are sepsis or systemic inflammatory response syndrome (SIRS), described as the set of clinical signs and symptoms that appears as a consequence of the activation of a systemic immune response, and multisystem organ dysfunction (MOD).[158–160]

The theory that has been extensively used for many years to explain this process is the so called “gut-origin sepsis”. Indeed, a functional alteration of the intestinal barrier gives rise to a pathological BT that turns the intestine into the key organ in the origin of infectious complications, development of SIRS, MOD and death through the entrance of bacteria and their toxic products to the systemic circulation (**Figure 6**).[160–162]

Increased knowledge in inflammatory process as a potential cause of clinical symptoms introduced a new perspective in the "gut-origin-sepsis" theory.[163] Indeed, the theory in which translocating bacteria were the direct causative factor of injury has evolved to an induction of the gut-associated lymphoid tissues. This induction activates a cascade of inflammatory responses mediated by cytokines and other nonbacterial pro-inflammatory factors like oxygen free radicals, triggering and maintaining systemic inflammation.[161–163]

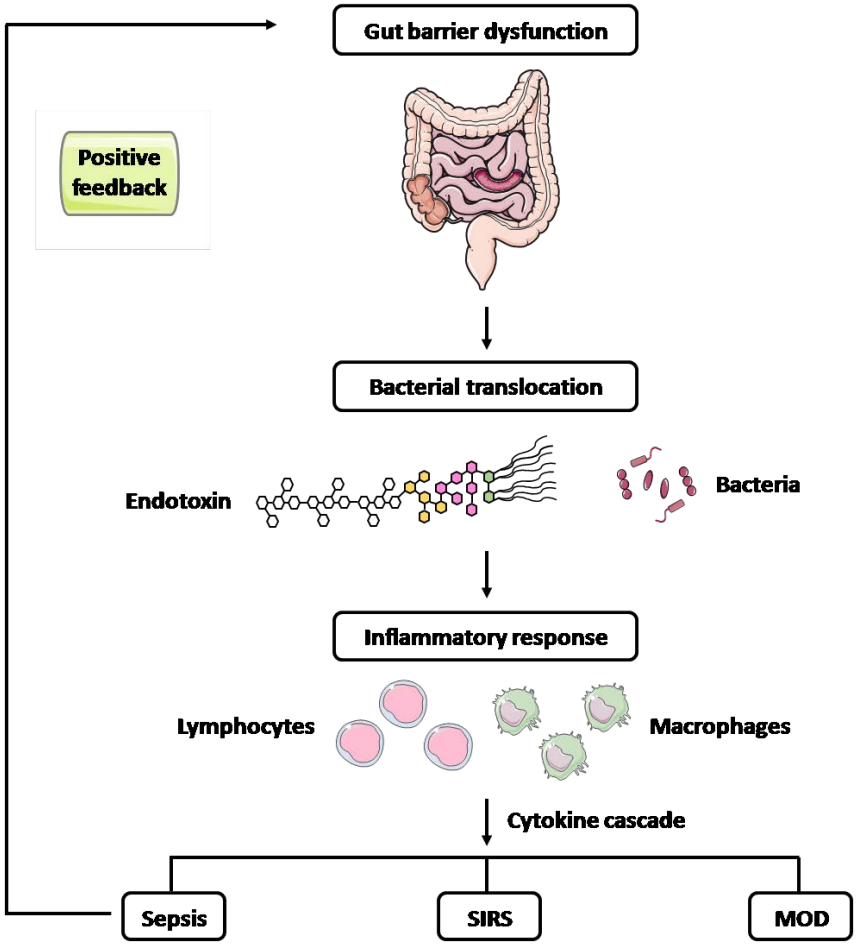


Figure 6. The gut origin of sepsis hypothesis. Functional alterations of the intestinal barrier lead to a pathological BT that turns the intestine into the key organ in the origin of complications

4. CIRRHOSIS

4.1. EPIDEMIOLOGY AND AETIOLOGY

Cirrhosis is an important public health problem, being one of the significant causes of morbidity and mortality in developed countries. It has been estimated that liver cirrhosis would be responsible for around 1.8% of deaths in Europe (170.000 per year)[164], while in the United States, it would be around 60.000 deaths per year representing the 12th leading cause of death overall.[165]

The causes that produce liver diseases are mainly preventable, and include alcohol abuse considered as the major risk factor, viral hepatitis B and C (HBV and HCV) and factors related to metabolic disorders such as overweight and obesity.[164] Other causes of liver disease are those which could be triggered by drugs or toxins, autoimmune hepatitis, Wilson's disease or hemochromatosis.[166]

In the last years, there has been a small decrease in cirrhosis mortality in developed countries, especially in Southern Europe. Surely, this trend is due to HBV vaccination, a decrease in alcohol consumption and in the transmission of HCV, and the development of effective treatments against HCV. However, cases of cirrhosis could be maintained in the coming years due to the continued high alcohol consumption and the increase in metabolic disorders.[164]

The knowledge of the disease's aetiology could help to take preventive measures, predict complications and improve management in the treatment of underlying diseases.[167]

4.2. PATHOPHYSIOLOGY AND CLINICAL COURSE

Until recently, cirrhosis was clinically seen as the point of no return of liver diseases, which may entail the need for a liver transplant in severe cases of liver failure. Recently, this perception of end-stage has changed, in part due to the decrease in mortality. Histologically, a change in the terminology has been proposed, switching to "advanced liver disease" as the new term of cirrhosis to emphasise that it could be a dynamic process with unsteady prognosis.[136]

Cirrhosis could be defined in different ways depending on the context from which it is being studied. Within the histopathological context, it is described as the change from a normal

structure of the liver lobules into an abnormal architecture of the liver where the nodules are delimited by fibrous tissue interfering with vascular connection.[168] Hemodynamically speaking, its definition could be based on the presence of portal hypertension that is considered as the most critical consequence of cirrhosis and holds most of its clinical complications. However, the most recognised is the clinical context that allows us to differentiate between compensated and decompensated stages.[169]

What is clear is that cirrhosis is a progressive and chronic condition that appears as a result of an hepatic inflammation followed by a necrosis of the parenchyma and activation of hepatic stellate cells causing fibrogenesis and leading to cellular dysfunction and portal hypertension.[136,168,170]

The clinical course of cirrhosis begins with an asymptomatic phase called compensated cirrhosis until it reaches the decompensated stage in which certain clinical complications appear, such as ascites, sepsis, gastrointestinal bleeding due to PHT, encephalopathy, jaundice, SBP or refractory ascites among others. However, an acute-on-chronic liver failure (ACLF) may occur at any disease state and it is associated with high mortality.[170,171]

Throughout these years, and taking into account histological, clinical, hemodynamic, and biological parameters, a chronic liver disease classification has been proposed in order to subdivide the clinical course in different stages as summarised in **Figure 7**. This classification is comprised from the initial state of the disease (Stage 1) defined by a compensated cirrhosis without varices until the decompensated stages (Stages 3 and 4) characterised by the appearance of complications.[169] Nevertheless, recently a new perspective in the stratification of the disease has been introduced. A further decompensation state, characterised by refractory ascites, renal and circulatory dysfunction or persistent encephalopathy among others, is considered as another stage by some authors.[170]

Moreover, cirrhosis is the most important risk factor for the development of hepatocellular carcinoma, which can develop at each stage.[167,172]

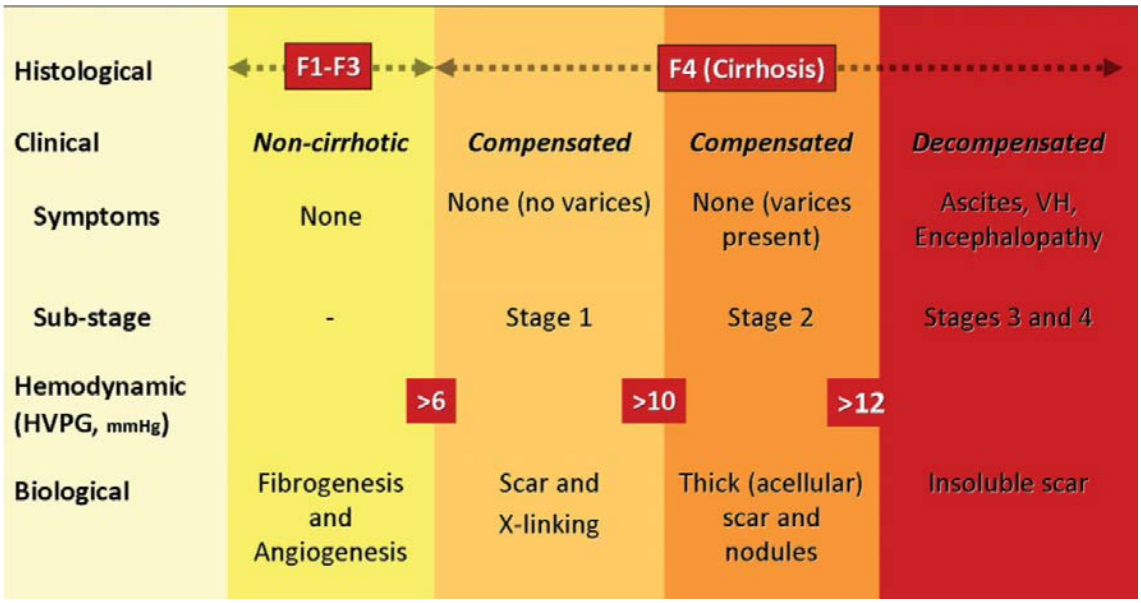


Figure 7. The different states of cirrhosis. Classification proposed to subdivide the clinical course of liver cirrhosis taking into account histological, clinical, hemodynamic, and biological parameters. This figure is a copy of Garcia-Tsao *et al.*, Hepatology, 2010.

Bacterial infections are well known to be involved in the worsening of cirrhosis, being critical in decompensated stages. They are considered one of the major causes of morbidity and mortality in these patients.[167,173,174] Hepatic function impairment together with immunological defects and the consequent increase in bacterial translocation, are the main mechanisms involved in the pathogenesis of bacterial infections.[174,175] With infection, a systemic inflammatory response syndrome may occur resulting in sepsis, renal failure, encephalopathy, and death.[174]

4.3. BACTERIAL TRANSLOCATION IN CIRRHOSIS

As mentioned, one complication of cirrhosis is infections, among which SBP, urinary tract infection, pneumonia and cellulitis predominate.[175] Most of them are caused by gram-negative bacteria, with a suspicion of enteric origin and therefore making the passage of bacteria through the epithelial barrier reaching the MLN, a possible mechanistic explanation of infections.[153]

Indeed, in cirrhosis there is a global alteration of the epithelial barrier function, including impairments in barrier permeability and immune system, which favours the appearance of a pathological BT.[117]

In terms of changes in the intestinal microbiota they could be quantitative, such as SIBO described in previous sections, or they could be qualitative. Dysbiosis with an increase in Enterobacteriaceae has been found as the most remarkable characteristic.[176]

Moreover, a disruption of the homeostasis in the epithelial barrier can lead to an increase of its permeability and therefore, allow the passage of luminal bacteria and their products. In disease condition, the components of the barrier are affected; for instance, in patients with cirrhosis a change in the characteristics of the mucus layer as well as in the composition of the microbiota associated with this layer has been seen.[177] In cirrhotic rat models a dysfunction of the Paneth cells with its consequent decrease in the release of antimicrobial peptides has also been observed.[178] Moreover, taking into account the secretory component, in patients with cirrhosis a reduction of IgA concentration has been described in faeces as well as in mucosal layers.[179]

In addition, in cirrhosis there is an increase in intestinal permeability secondary to structural and functional changes of the epithelial barrier derived from the disease itself. PHT induces those changes that include a widening of intercellular spaces, vascular congestion, oedema, fibrous tissue proliferation, decreased villous/crypt ratio, thickened muscularis mucosae, and inflammatory changes[180,181] with an incremented oxidative stress followed by a consequent worsening of mucosal gut damage.[182]

As previously mentioned, the immune system is also playing a critical role in the promotion of BT. The immune dysfunction situation in which cirrhotic patients are found, is known as cirrhosis associated immune dysfunction syndrome.[183] It is characterised by an alteration in the innate and adaptive immunity causing a simultaneous occurrence of opposed processes, depression and overstimulation. On one side, a dysfunction in the GALT avoids an effective clearance of bacteria and their products favouring high levels in systemic circulation.[144] On the other hand, it appears an inflammatory response with a release of proinflammatory cytokines, like TNF-α, interleukins and NO which can cause intestinal and liver tissue damage, perpetuating BT and the mechanisms that are involved in its physiopathology.[167,183,184]

As specified, the existence of a pathological BT is a key point in the development of complications in cirrhosis and is related to the stage of liver failure being more significant in advanced phases of the disease.[153,177]

So, the hypothesis proposed by Wiest *et al*. would be that small but constant increases in paracellular translocation of bacterial products in initial stages of the disease, would cause an activation of the immune response leading to a release of cytokines and reactive oxygen species. Thus, this release would cause a greater disruption of the intestinal barrier and therefore a perpetuation of BT. In addition, these mechanisms would entail an adaptation and tolerance of the immune system to the intensified passage of bacteria. Finally, in the more advanced stages, or decompensated cirrhosis, there would be an increase of the intestinal barrier permeability allowing transcellular passage, which along with the decrease in antimicrobial peptides secretion and an acceleration of SIBO makes all a vicious circle (**Figure 8**).[177]

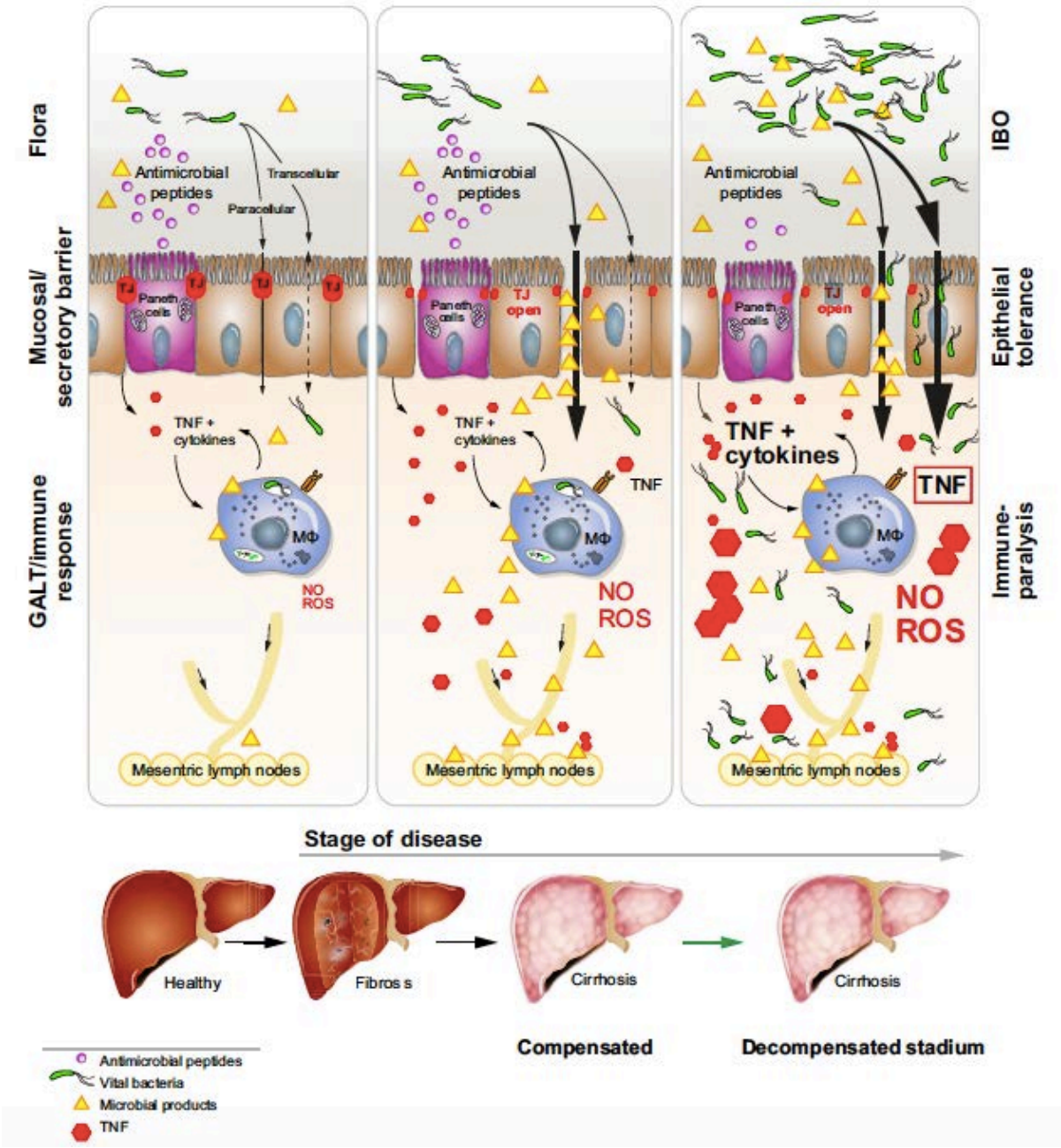


Figure 8. Hypothesis of the involvement of pathological BT in the progression of liver disease. Alterations of the gut microbial composition could favour BT and thus, activate an immune response. The subsequent inflammatory response, creates a feedback in which BT promotes its own causative mechanisms. This figure is a copy of Wiest *et al.*, Journal of Hepatology, 2014.

5. METHODS TO STUDY MICROBIAL TRANSLOCATION

The presence of BT is associated with an advanced stage of cirrhosis and is considered as a key factor for the appearance of complications,[153] but it is not yet known exactly up to what extent

it is involved and the exact mechanism through which it acts. For this reason, there is a growing interest in the study of BT although its evaluation is not entirely direct and easy.

Among **direct methods,** we find the direct determination of bacteria in the MLN through culture. It has been and may still be one of the most used techniques in experimental and clinical studies, although access to MLN in humans is very limited. In addition, it is a technique that could be underestimating BT, since traditional culture techniques allow the culture of only a minority of intestinal bacteria. Thus, a positive culture would confirm BT while a negative one would not rule out its presence.[112,158,160]

Another method included in this section is the one that uses radioactively labelled bacteria and is capable to detect non-viable bacteria.[112]

Among **indirect methods,** there are those that allow a less invasive detection of BT. Within the more classic techniques, detection of intestinal bacteria through culture of portal or peripheral blood and endotoxins like lipopolysaccharide (LPS) in peripheral blood is performed. Nowadays, LPS has been replaced by (lipopolysaccharide binding protein) LBP a protein produced in response to bacteraemia or endotoxemia. However, the advances in molecular biology have allowed progress towards newest methods based on the detection of microbial DNA through polymerase chain reaction (PCR).[112,153]

Nevertheless, the study of intestinal permeability is still widely used for the determination of BT which can be evaluated through a wide variety of techniques. Urine determination of non-metabolizable sugars such as lactulose and mannitol are one of the most used methods. Other useful non-digestible markers could be radioisotopes and polyethylene glycols. [144,185] Even so, an increase in intestinal permeability, a well-known factor that favours the appearance of BT, is a reliable proof that this phenomenon is occurring.

6. PRELIMINARY OBSERVATIONS

Until the appearance of new molecular techniques the phenomenon of bacterial translocation was considered a dichotomic and mostly monomicrobial phenomenon.[186] With these advances, some studies were able to detect more than one bacterial species in MLNs of healthy mice suggesting that BT was a polymicrobial phenomenon.[187]

In addition, a dysbiosis in faeces of cirrhotic patients has been demonstrated,[188] but there were no data regarding MLNs, one of the essential components that trigger the immune response against pathogens and its consequent inflammatory response.

The need of an appropriate animal model to study different pathologies sometimes is due to the difficulty of analysing certain type of samples such as MLNs. In the case of cirrhosis, one of the most widely used models is based on the induction of chronic liver damage in rodents through the administration of carbon tetrachloride (CCL_4).[189]

Therefore, and taking into account the current knowledge, there was a necessity to evaluate the microbial composition of the MLN in a rat model and its relationship with the immune response.

Finally, and as presented in **Annex 1**, the study carried out by our group was the first to report a high microbial diversity in MLNs, in both control and cirrhotic rats, with Proteobacteria being the most abundant phylum, suggesting a possible greater ability of these bacteria to attach and penetrate the intestinal barrier. These results confirmed that bacterial translocation is a constant, physiological and polymicrobial phenomenon. In addition, dysbiosis was demonstrated in the MLN of cirrhotic rats with a decrease in the diversity as well.

These promising preliminary observations led us to consider the possibility of studying in more depth the phenomenon of translocation, in both rats and humans.

HYPOTHESIS

HYPOTHESIS

It is known that gut microbiota plays an important role in cirrhosis, bacterial translocation being a key factor in the pathogenesis and its complications. Moreover, it has been shown that a dysbiosis, and the passage of bacteria reaching the liver and other extra-intestinal sites through the portal vein, is associated with the severity of the disease.

According to the hypothesis proposed by Wiest *et al*.[177], we consider that alterations of the gut microbial composition could favour BT and thus, activates an immune response and the release of cytokines with the subsequent inflammatory response, creating a feedback in which BT promotes its own causative mechanisms.

However, very little is known about the microbiome of extra-intestinal sites in patients with cirrhosis or the underlying mechanisms that involve gut microbiota in the prognosis. Therefore, more in-depth studies are needed for a better understanding of the mechanisms of this phenomenon in order to evaluate its real relevance in cirrhosis, and to improve the design of more appropriate prognostic and therapeutic strategies.

OBJECTIVES

OBJECTIVES

MAIN OBJECTIVE

The main objective of this project was to understand up to which point microbial translocation is involved in cirrhosis. In order to obtain an appropriate response, two studies were carried out with the following specific objectives:

SPECIFIC OBJECTIVES

Study 1. Alteration of the serum microbiome composition in cirrhotic patients with ascites

This study sought to: (a) characterise the microbiome of serum and faecal samples of patients with cirrhosis and compare them with those of healthy controls; (b) define the serum microbiome associated with severity of liver disease; and (c) identify the microbiome of ascitic fluid.

Study 2. Sequential changes in the mesenteric lymph node microbiome and immune response during cirrhosis induction in rats

This study sought to (a) investigate the spatial and temporal changes of the composition and function of the microbiome in a cirrhosis rat model; (b) evaluate changes of the microbiome as they relate to the progression of cirrhosis; and (c) assess the immune modulation by the microbiome detected in MLNs.

METHODS

METHODS

1. Ethical Committees approval for human and animal studies

All experiments were performed in accordance to ethical guidelines. The protocol for the cirrhosis study in humans was submitted and approved by the Clinical Research Ethics Committee of the Hospital de la Santa Creu i Sant Pau, Barcelona. All the subjects received information concerning their participation in the study and gave written informed consent.

The protocols for the studies performed with animal models were approved by the Animal Research Committee at the Institut de Recerca of Hospital de la Santa Creu i Sant Pau, Barcelona, in the case of cirrhosis study. They were further approved by the Department of Agriculture, Livestock and Fisheries of the Generalitat de Catalunya (Departament d'Agricultura, Ramaderia i Pesca). Animal care complied with the criteria outlined in the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals Institute for Laboratory Animal Research Division on Earth and Life Studies, Washington, DC, USA).

2. Sample collection and storage

2.1. Sample collection from humans

After deposition of faecal matter, homogenisation was done using a spatula by the subject and samples were immediately frozen in their home freezer at -20°C. Frozen samples were later brought to the laboratory in a freezer pack, where they were stored at -80°C until further processing.[190] Aliquots of 250 mg were performed on solid CO_2 (dry ice) to maintain the frozen status of the sample avoiding the degradation of nucleic acids.

2.2. Sample collection from an animal model of cirrhosis

The study was carried out in the animal house at Hospital de la Santa Creu i Sant Pau. Male Sprague-Dawley rats weighing 35–49 g were purchased from Harlan Laboratories (Indianapolis, Ind., USA) and provided by Research Models and Services Production (Udine, Italy). After the rats were weaned from their mothers, they were fed a rodent chow diet (2018S; Teklad, Madison, Wisc., USA). After a 1-week quarantine, all animals were isolated in a sterilised cage in order to avoid the transmission of

microbiota between individuals. All rats drunk sterilised water and were fed an autoclaved chow diet. Animals were kept at stable conditions of temperature (21°C) and exposed to a 12-hour light:12-hour dark cycle.

When rats reached a weight of 200 g, they were administered weekly doses of CCl_4 (Sigma-Aldrich, St. Louis, Mo., USA) intragastrically using a sterile pyrogen-free syringe (ICO plus 3 Novico Medica, S.A., Barcelona, Spain] with an attached stainless-steel animal feeding tube (Popper and Sons, New Hyde Park, N.Y., USA) without anaesthesia. The initial dose of CCl_4 was 20 µl, and subsequent doses were adjusted based on the change in body weight 48 h after the previous dose, as reported previously [18, 19]. When cirrhotic rats presented ascites, the dose of CCl_4 was maintained at 40 µl. Non-cirrhotic rats receiving tap water were used as controls.

Laparotomy was performed at the corresponding weeks (week 6, week 8 and week 10) in all cirrhotic and control rats and in a last group when ascites was suspected by the increase in abdominal girth and confirmed by paracentesis as shown in **Figure 10**. They were anesthetised with 10 mg/kg xylazine (Rompun®; Bayer, Kiel, Germany) and 50 mg/kg ketamine (Ketolar®; Parke-Dawis, Madrid, Spain) in sterile conditions. In brief, the abdominal fur was removed with a depilatory cream and the skin was sterilised with iodine. The abdomen was then opened via a 4-cm median incision, and the remaining fluid was removed. Samples of ascitic fluid (and pleural fluid if present) were collected for bacterial culture. Ten MLNs were aseptically and randomly collected from the ileocecal area, weighed, homogenised in sterile saline solution and stored at -80°C for later analyses. Blood was collected from the vena cava into a sterile EDTA-containing BD Vacutainer ® tube (BD Biosciences, San Jose, Calif., USA) without additives, centrifuged and stored at -80°C until later analysis. Liver and spleen tissue were sampled for histological evaluation and bacterial culture and frozen at -80°C. Rats were then euthanised with intravenous sodium thiopentate (Pentothal®; Abbott Laboratories).

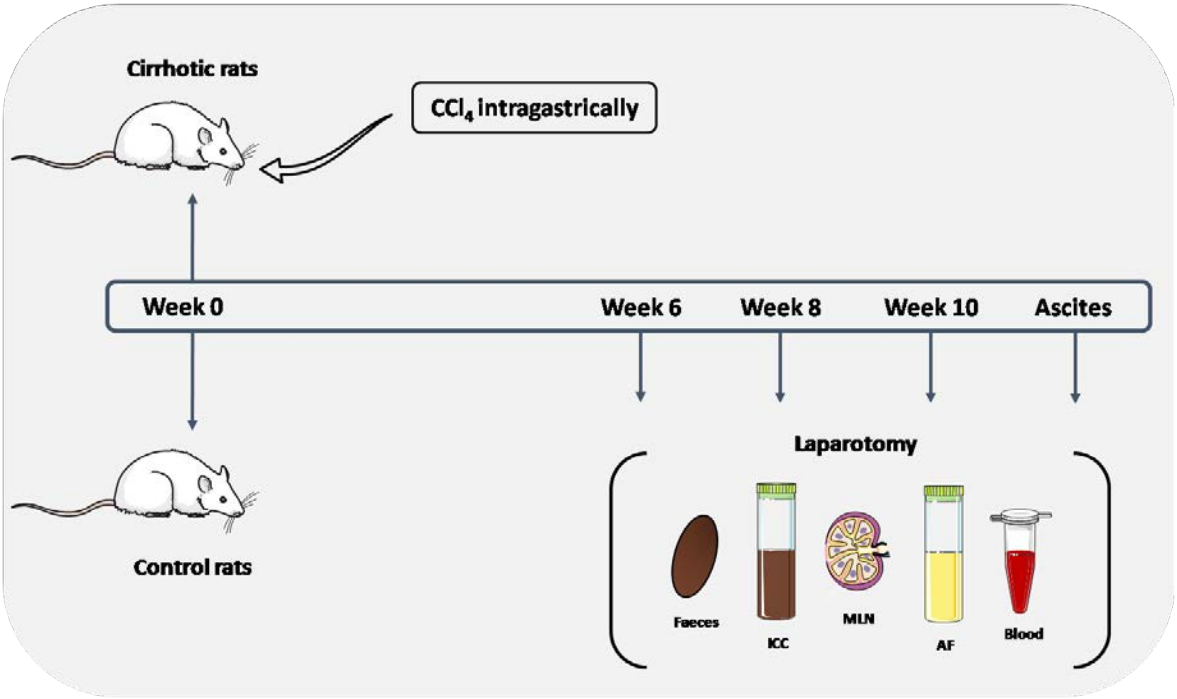


Figure 10. Schematic representation of the experimental design for the animal model. Laparotomy performed at different weeks simulating the progression of the disease (compensated and decompensated stages) in both, cirrhotic and control groups.

3. DNA extraction

3.1. DNA extraction from faeces

Before starting the extraction procedure, 800 mg of 0.1mm Zirconia/Silica beads, previously sterilised with UV, were put in the tubes. To chemically lyse the samples, 250 µl of 4 M guanidine thiocyanate, 40 µl of 10% N-lauroylsarcosine and 500 µl of 5% N-lauroylsarcosine were added and incubated at 70°C for 1 hour. Further mechanical disruption was carried out using a Beadbeater (Biospec Products©) to ensure disruption of cell wall of gram positive bacteria and avoid a bias during the bioinformatics analysis as shown by a previous study carried out at our laboratory (**Annex 2**).[191] Poly Viny Poly-Pyrrolidone (PVPP) was added in multiple washing steps to precipitate and discard aromatic molecules such as nuclei debris, cellular debris and proteins. To clear lysates, enzymatic digestion of RNA was performed, and resulting DNA was precipitated and further ethanol-purified.[192] Pure DNA was re-suspended in 200µl Tris-EDTA buffer.

3.2. DNA extraction from low biomass samples

Microbiological habitat is not restricted only to faeces, but there is other type of intestinal and extra intestinal tissues with a very low microbial load considered as "low biomass". So as to be able to obtain comparable data within the same study and between studies, it is necessary to standardise methods. That is why, and based on our experience, we modified and adapted the DNA extraction protocol for faeces to “low biomass” samples such as swabs, biopsies, MLNs and fluids.

Specific quantity for each sample was prepared and stored in specific tubes as specified in **Table 1**. Before starting the extraction, each sample type was submitted to a distinct pre-treatment:

Sample Type	Recipient	Quantity of starting sample
◆Swabs	2 ml screw cap tube	1 unit
◆Biopsies	2 ml screw cap tube	10 - 40 mg
◆MLNs	2 ml screw cap tube	10 - 40 mg
◆Fluids	15 ml centrifuge tube	5 ml

Table 1. Description of the specificities that have to be taken into account for each sample type prior the DNA extraction

Swabs No pre-treatment is needed

Biopsies Add 180 µl of ATL buffer from Qiagen (ATL, ref. 939016) and incubate at 37°C for 30 min. Add 25 µl of proteinase K and incubate at 56°C until complete dissociation.

MLNs Add 180 µl of the ATL buffer and incubate at 37°C for 30 min. Add 25 µl of proteinase K and incubate at 56°C for until complete dissociation.

Fluids Centrifuge the 15 ml tube during 15 min at 20817g and remove the supernatant.

After this pre-treatment and the addition of the 800 mg of 0.1mm Zirconia/Silica beads, previously sterilised with UV, the chemical lysis of the samples was carried out with 250 µl of 4 M guanidine thiocyanate, 40 µl of 10% N-lauroylsarcosine and 500 µl of 5%

N-lauroylsarcosine and an incubation at 70°C for 1 hour. Further mechanical disruption was carried out using a Beadbeater (Biospec Products©). Poly Viny Poly-Pyrrolidone (PVPP) was added in multiple washing steps to precipitate and discard aromatic molecules such as nuclei debris, cellular debris and proteins. To clear lysates, enzymatic digestion of RNA, in a higher concentration than for faeces, was performed, and resulting DNA was precipitated and further ethanol-purified. Pure DNA was re-suspended in 30µl Tris-EDTA buffer (**Figure 9**).

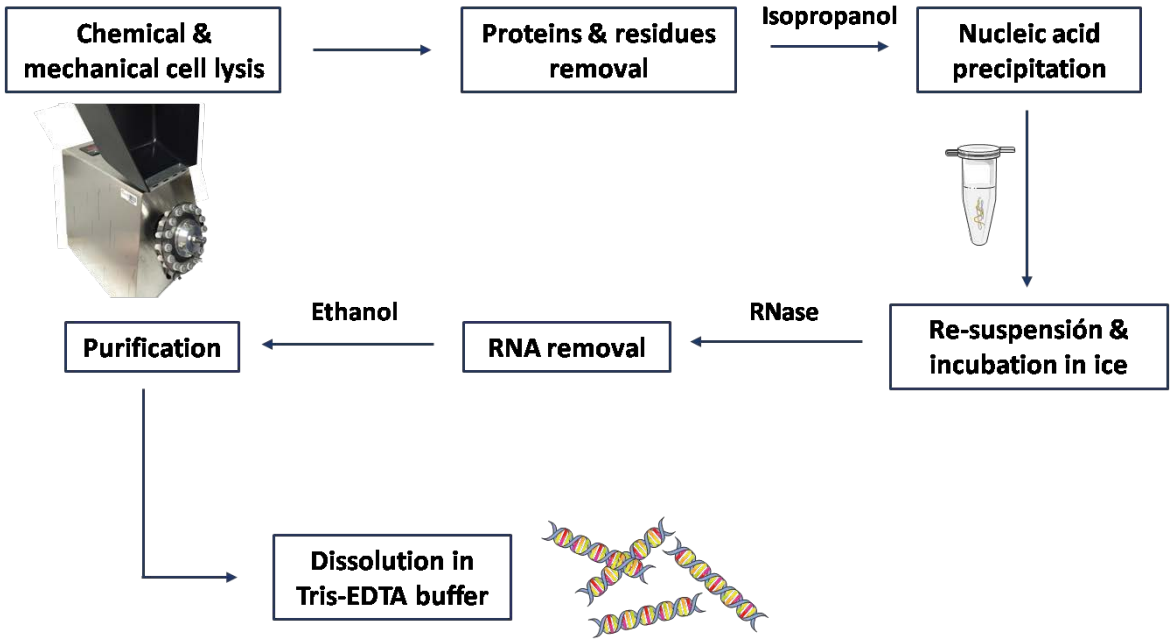


Figure 9. Overview of the extraction method. All the steps performed during the extraction manual protocol are summarised in the figure to better understand the principles of the method used.

An equivalent of 1 mg of each sample was used for DNA quantification using a NanoDrop ND-1000 Spectrophotometer (Nucliber©). DNA integrity was examined by micro-capillary electrophoresis using an Agilent 2100 Bioanalyzer with the DNA 12,000 kit, which resolves the distribution of double-stranded DNA fragments up to 17,000 bp in length.

4. Quantitative PCR

For determining microbial load or abundance, a real time quantitative Polymerase Chain Reaction (qPCR) was performed with the amplification of 16S rRNA gene which is widely used

as a phylogenetic marker.[193] For this purpose, the V4 region of the 16S rRNA gene was amplified using universal primers: Forward primer V4F_517_17 (5'-GCCAGCAGCCGCGGTAA-3') and Reverse primer V4R_805_19 (5'-GACTACCAGGGTATCTAAT-3'). PCR reactions were performed in optical-grade 96-well plates in a final volume of 25µl, containing 100nM of each primer and Power SYBR Green Master Mix (Applied Biosystems©). A minimum of 5-log cycle standard was maintained to be able to plot the sample quantities against a known standard curve. Each sample, standards and negative controls were amplified in triplicates, from which mean values were calculated for final statistics. qPCR experiments were also done in duplicates to ensure accuracy and the mean values of both experiments were taken into account. All reactions were performed with the 7500 Fast Real-Time PCR System (Applied Biosystems©). Reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analysed using Sequence Detection Software version 1.4, supplied by Applied Biosystems©.

5. 16S rRNA gene amplification for Illumina sequencing

For microbiome composition profiling, the hyper-variable region (V4) of the bacterial and archaeal 16 S rRNA gene was amplified by PCR. The 5' ends of the forward (V4F_515_19: 5' -GTGCCAGCMGCCGCGGTAA-3') and reverse (V4R_806_20: 5' -GGACTACHVGGGTWTCTAAT-3') primers targeting the V4 region of 16S gene were tagged with specific sequences for Illumina® MiSeq Technology as shown in **Table 2**. 12 base paired Golay codes were specified downstream of the reverse primer sequence (V4R_806_20) to allow multiplex identification of individual samples.[194,195]

PRIMER TYPE	SEQUENCE 5' → 3' ILLUMINA FLOWCELL - BARCODE - ADAPTER - LINKER - V4 REGION
Forward_illumina	AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
Reverse_illumina	CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

Table 2. Details of the primers used 16S rRNA gene amplification for Illumina sequencing

Standard PCR using 0.75 units of *Taq* polymerase (AmpliTaq Gold, Life Technologies®) and 20pmol/µl of the forward and reverse primers (IDT Technologies®) in a final volume of 50 µl was run in a Mastercycler gradient (Eppendorf©) at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec, and a final cycle of 72°C for 10 min.

6. Agarose gel and purification

1% agarose gel stained with ethidium bromide was performed and run in 1x Tris Acetate EDTA (TAE) buffer. 5µl of PCR product was mixed with 6x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and placed it in the wells of solidified agarose gel along with 100bp DNA Molecular Weight Marker XIV (Roche©). Immediately, the gel electrophoresis was run in an electrophoresis tank (Bio- Rad©) at about 90-100V for 35-45 minutes to visualise amplicon bands in a Gel Doc XR+ system (Bio-Rad©). The appearance of bands, confirmed that amplicons had been generated during PCR amplification. The absence of bands could be explained by the presence of few bacterial DNA in the sample (especially for low biomass samples) or by the presence of PCR inhibitors added throughout the procedure (in those cases more diluted DNA samples were needed to get amplicons).

Once the amplification was confirmed, the samples were purified using the QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain) according to manufacturer's instructions, and further quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber©).

For low biomass samples an extra purification step was needed to eliminate salts, primer-dimers and dNTPs more efficiently. Specific DNA fragments are bound to magnetic beads using the HighPrep™ PCR (MAGBIO) according to manufacturer's instructions.

7. Illumina sequencing

As described by the Illumina website, the sequencing process consists of:

[https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf]

"Sequencing templates are immobilised on a proprietary flow cell surface designed to present the DNA in a manner that facilitates access to enzymes while ensuring high stability of surface

bound template and low non-specific binding of fluorescently labelled nucleotides. Solid-phase amplification creates up to 1,000 identical copies of each single template molecule in close proximity. Sequencing by synthesis technology uses four fluorescently labelled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. During each sequencing cycle, a single labelled deoxyribonucleoside triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Since all four reversible terminator-bound dNTPs (A, C, T, G) are present as single, separate molecules, natural competition minimises incorporation bias. Base calls are made directly from signal intensity measurements during each cycle, which greatly reduces raw error rates" (**Figure 10**).

Amplicon pools of equal concentration were diluted to 2nM and spiked with 15-30% denatured PhiX. Then, denatured templates were further diluted to 5ρM and subsequently combined to give an 85% 16S rRNA gene amplicon library and 15% PhiX control pool. Sequencing was performed with the use of a MiSeq (Illumina Technologies©) Reagent Cartridge (300-cycle PE kit) where appropriate index and sequencing primers were added.

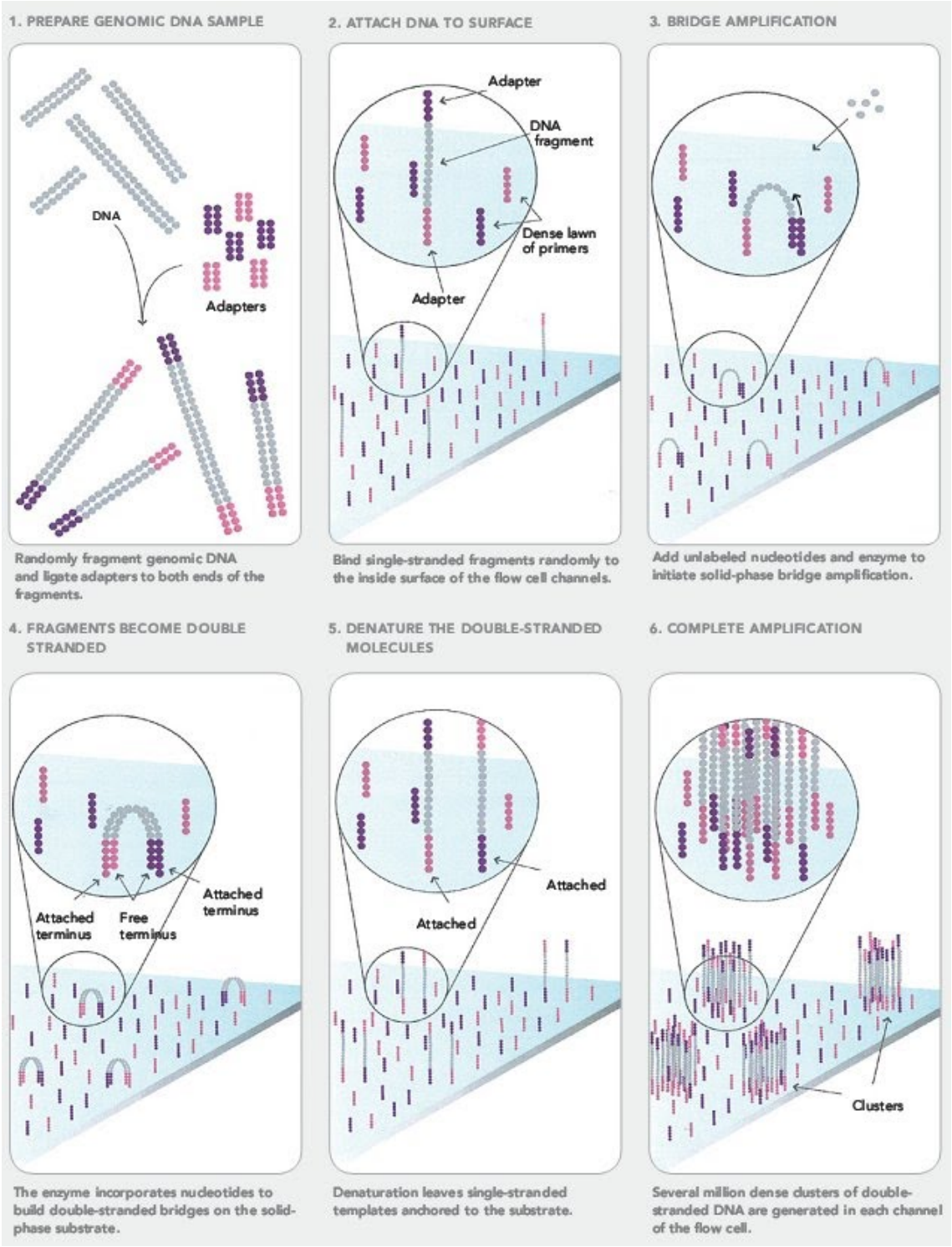


Figure 10. Diagram of Illumina sequencing technology

8. Sequence analysis

The bioinformatics pipeline used for data analysis was Quantitative Insights Into Microbial Ecology (QIIME) [www.qiime.org].[196] Briefly, the process is divided into two stages: upstream

and downstream. The former process includes the raw data processing with appropriate file generation for the subsequent microbial analysis carried out during the downstream step (**Figure 11**).

Prior the upstream analysis, a metadata containing sample identifiers, barcodes, primer sequence, time point, sample status, clinical information of the subjects involved in the studies and other additional information of the samples needed for the analyses was properly filled out.

Then, the raw data sequences along with metadata were loaded into the QIIME 1.8.0 or 1.9.1 pipeline[195] and a verification of the quality of the sequence files provided by the sequencing platform was done filtering out reads with a Phred score less than 20.[197] Throughout the sequencing process, errors can occur causing an overestimation in the number of Operational Taxonomical Units (OTUs).

In order to remove the primers and the barcodes (short-DNA sequences unique to each sample) used during the PCR process from raw sequences, a demultiplexing step was run. Then, using a pick-OTUs protocol (UCLUST or USEARCH algorithms) similar filtered sequences were clustered into OTUs based on a 97% similarity threshold. Chimeric sequences were removed using ChimeraSlayer or UCHIME (**Table 3**).

Taxonomy was assigned using the Basic Local Alignment Search Tool (BLAST) with combined datasets encompassing Greengenes and PATRIC databases using PyNAST that generated an OTU table containing all the OTUs with the assigned taxonomy and abundances for each sample.

Finally, the downstream analysis was carried out in which multiple rarefactions were applied. The output files were used to compute alpha diversity which allows the estimation of microbial richness and beta diversity defined as the amount of variation in species composition between samples.[198] For beta diversity, defined as the amount of variation in species composition between samples, weighted and unweighted UniFrac methods were used to generate phylogenetic distance matrices that were later used for clustering samples in hierarchical cluster trees with Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Principal Coordinate Analysis representations (PCoA).

The summarise taxa feature was used to classify taxa from the Domain to the Species level that were subsequently used for study and sample specific statistical analyses.

Furthermore, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) version 0.9.1 characterised by its capacity to predict functions from 16S data and a reference genome database[199] was used to predict functions in the animal model study.

	FIRST STUDY	SECOND STUDY
QIIME version	1.8.0	1.9.0
OTU clustering	Uclust	Usearch
Chimera removal	ChimeraSlayer	UCHIME

Table 3. Summary of the updated algorithms and programs used during the different steps of the sequence analysis.

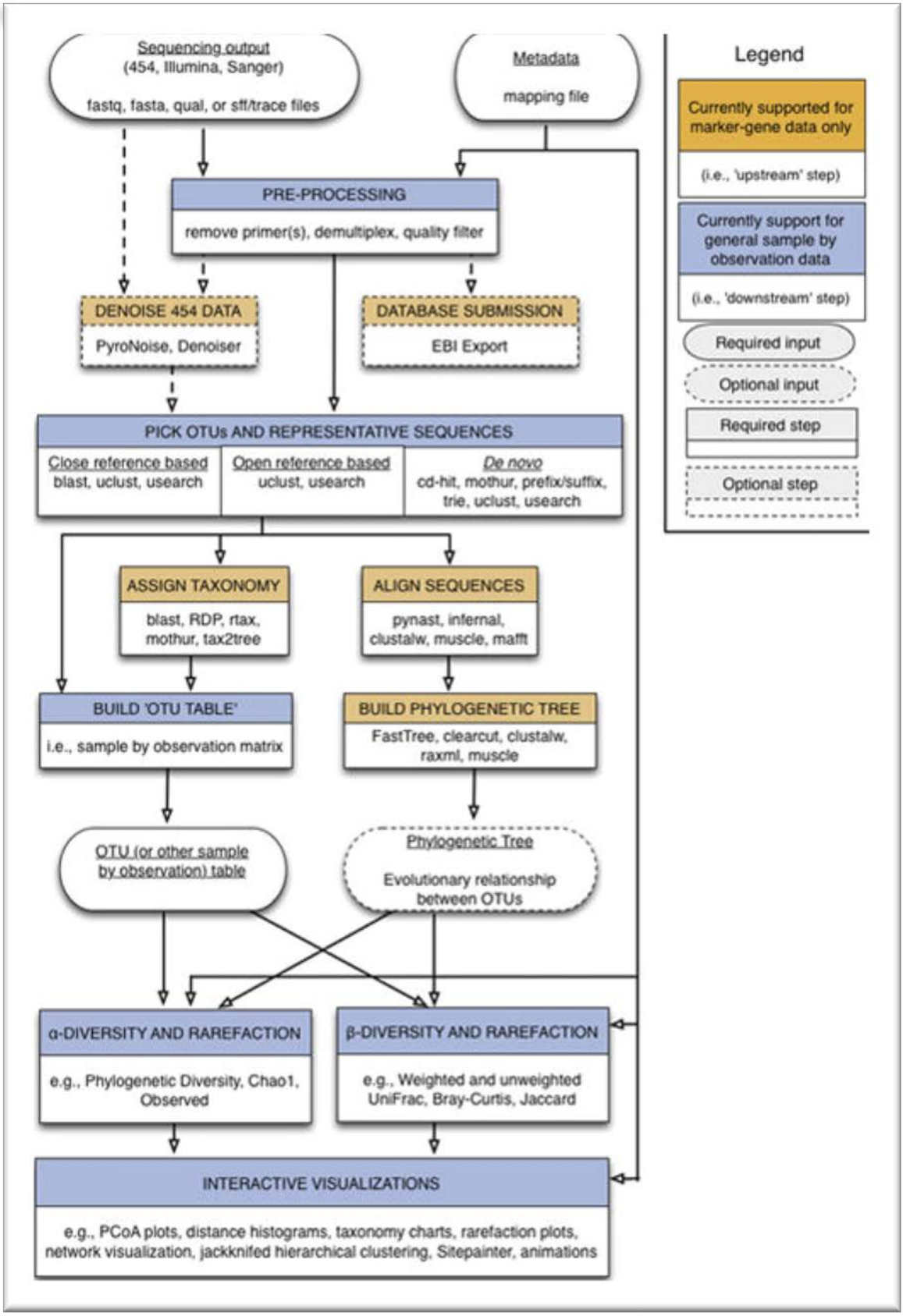


Figure 11. Proposed scheme for analysis of data generated by Illumina

9. Statistical tests

To check normality of the distribution of the data, both Saphiro-Wilk test[200] and Agostino-Pearson test[201] were used.

Mann Whitney test is a non-parametric analysis that compares two unpaired groups.[202] This test only compares two groups; to compare three or more groups, we used the Kruskal-Wallis test. Kruskal-Wallis test is a non-parametric test that compares the medians of three or more unpaired or unmatched groups, which may have different sample sizes, for testing whether the samples originate from the same distribution.[203]

If the data were paired or related, then Wilcoxon matched pairs test was used.[204] This is a non-parametric test that compares two paired groups where the factor that caused a difference between the paired values is too different in only one of them. When comparing more than 2 paired groups the Friedman test was used.

Parametric data with normal distribution were compared using the Student's t test for paired or unpaired data.

To test for differences in microbial communities we performed a non-parametric permutational multivariate analysis of variance (PERMANOVA) called Adonis test. So as to evaluate possible relationships between microbial genera and variables in clinical data the non-parametric Spearman's rank correlation coefficient was used.[205]

Characteristics of patients were compared using the Fisher's exact test which is a statistical significance test used in the analysis of contingency tables that include two or more variables.[206]

When possible, False Discovery Rate (FDR) corrected p-values were taken into account to consider significant results sometimes expressed in the text as "q" to avoid the type I error in multiple comparisons (FDR or $q<0.05$ was considered significant).

10. Determination of lipopolysaccharide binding protein levels

Determination of the levels of LBP to assess exposure to bacteria and their endotoxins as an index of bacterial translocation[207] were carried out in serum samples using specific enzyme-linked immunosorbent assay (ELISA) (Biometec GmbH, Greifswald, Germany) according to the

manufacturer's instructions. LBP was quantified with standard curves provided by the corresponding ELISA kit. The detection limit was 5 ng/mL.

11. Cytokine measurement

Determination of TNF-α, IL-6 and IL-10 cytokines was done in blood samples and IL-17 in MLNs by ELISA, according to the manufacturer's protocols (eBiosciences). Results are expressed as pg/ml in blood samples and the ratio IL-17 pg per mg of total protein. Limits of detection were 30 pg/ml for TNF-α, IL-6 and IL-17 and 15 pg/ml for IL-10.

CHAPTER 1

CHAPTER 1. CIRRHOSIS IN PATIENTS

"Alteration of the serum microbiome composition in cirrhotic patients with ascites"

ABSTRACT

The progression of cirrhosis is associated with alterations in the composition of the gut microbiome. To assess microbial translocation, we compared the serum microbial composition of patients with and without ascites and characterised the ascitic fluid microbiome using 16S rDNA high-throughput sequencing data. A complex and specific microbial community was detected in the serum and ascetic fluid of patients with cirrhosis but barely detectable in the serum of healthy controls. The serum microbiome of patients with ascites presented higher levels of lipopolysaccharide binding protein, a marker of microbial translocation, associated with higher diversity and relative abundance of Clostridiales and an unknown genus belonging to the Cyanobacteria phylum compared to patients without ascites. The composition of the faecal microbiome was also more altered in patients with than without ascites, confirming previous studies on faecal microbiome. We propose that alteration of the serum and faecal microbiome composition could be considered indicators of cirrhosis progression.

INTRODUCTION

Liver cirrhosis is a major cause of global health loss. In this regard, its incidence increased from 676,000 patients in 2008 to over 1 million in 2010[1]. It is the final phase of chronic liver disease, in which inflammation is associated with dying hepatic cells and fibrosis, leading to poor liver function and portal hypertension. Alterations in the gut microbiota, which represents the collective microbial cells present in the digestive tract, or its products, are linked to the progression of liver disease and the complications of cirrhosis[2]. Over the last decade, advances in molecular techniques and bioinformatics, as well as the exponential decrease in the cost of sequencing, have allowed comprehensive characterization of the composition and function of the gut microbial community. Using these techniques, recent studies on the gut microbiome have demonstrated an alteration of the composition of the stool microbial community in cirrhotic patients compared to healthy controls[3,4]. Furthermore, this level of alteration appears to be positively correlated with the severity of the disease[5].

More specifically, bacterial translocation has been suspected to play an important role in the pathogenesis and complications of cirrhosis. By administering green fluorescent protein (GFP)-labelled Escherichia coli orally to cirrhotic rats, Teltschik *et al*.[6] revealed the presence of bacteria not only in the intestinal lumen but also in MLNs and ascites. We also recently described that rat MLNs harbour a high microbial diversity[7]. However, very little is known about the microbiome of extra-intestinal sites such as the systemic circulation
and ascitic fluid in patients with cirrhosis.

This study sought to: (a) characterise the microbiome of serum and faecal samples of patients with cirrhosis and compare them with those of healthy controls; (b) define the serum microbiome associated with severity of liver disease; and (c) identify the microbiome of ascitic fluid.

METHODS

Ethical statement

The study included consecutive outpatients with cirrhosis treated at the Hospital de la Santa Creu i Sant Pau, a tertiary care hospital in Barcelona, Spain. The methods conformed to the Declaration of Helsinki and Guidelines for Good Clinical Practice in Clinical Trials and were carried out in accordance with the Clinical Research Ethics Committee of the Hospital de la Santa Creu i Sant Pau. All experimental protocols were approved by the same Ethics Committee. All patients received information concerning their participation in the study and gave written informed consent.

Patient information

Cirrhosis was diagnosed by clinical, analytical, and ultrasonographic findings or by liver biopsy. Exclusion criteria were the following: hospitalization in the previous month due to decompensation of cirrhosis; hepatocellular carcinoma or other neoplasia; alcohol intake in the previous 3 months; current infection or overt hepatic encephalopathy; marked symptomatic comorbidities (cardiac, pulmonary, renal, untreated active depression); treatment with antibiotics or non-absorbable disaccharides in the previous 3 months; and life expectancy of less than 6 months. Patients were carefully evaluated to exclude active infection when joining the study. Patients were classified into two groups, namely those with ascites and those without. The former group consisted of stable patients with refractory ascites attending the day hospital for regular therapeutic paracentesis. A group of age- and gender-matched healthy controls was included to compare their stool and blood microbiome composition with that of patients with cirrhosis.

Sample collection

Faecal samples were collected by the patients or controls as previously described[8]. Blood and ascitic fluid samples were collected in sterile conditions by peripheral vein puncture and during therapeutic paracentesis, respectively. For patients with cirrhosis, we performed routine blood analysis to assess the degree of liver failure, renal function, blood white cell count, and ascitic fluid neutrophil count to rule out ascitic fluid infection (SBP). Samples of blood and ascitic fluid were cultured in blood culture bottles (BactAlert®) to assess for microbial growth. Additional

samples of blood and ascitic fluid were collected in in SST™ Tubes (BD Vacutainer®) tubes and 15 ml centrifuge tubes respectively, and frozen at − 80°C until DNA analysis.

Lipopolysaccharide binding protein levels

Serum was tested for LBP concentration to assess exposure to bacteria and their endotoxins as an index of bacterial translocation[9,10], using specific ELISA (Biometec GmbH, Greifswald, Germany) according to the manufacturer's instructions. LBP was quantified with standard curves provided by the corresponding ELISA kit. The detection limit was 5 ng/mL.

DNA extraction, PCR amplification, and sequencing

We analysed the microbiome of samples from healthy controls (stool, n = 17; serum, n = 7) and cirrhotic patients (stool, n = 27; serum, n = 27; ascitic fluid, n = 11). In order to identify possible contamination in low-biomass samples and subtract the sequences of the potentially contaminated DNA generated during the extraction and PCR amplification, we introduced negative controls (blanks) during these two technical steps.

A frozen aliquot of faecal sample (250 mg) from each individual (n = 44) was subjected to genomic DNA extraction using a previously described method, referred to here as the "Godon" method.[8,11] Each sample was suspended in 250 µ l of guanidine thiocyanate, 0.1 M Tris (pH 7.5), 40 µ l of 10% N-lauroyl sarcosine, and 500 µl 5% N-lauroyl sarcosine. DNA was extracted by mechanical disruption of the microbial cells with beads. RNA was removed by the addition of 2 µ l of a 10-mg/ml solution of RNAase, and nucleic acids were recovered from clear lysates by alcohol precipitation. Twenty-seven and seven serum samples were collected from patients and healthy controls, respectively, and subjected to genomic DNA extraction using beads to disrupt the microbial cells followed by the QIAamp® DNA Blood Midi Kit (Qiagen, Madrid, Spain), following the manufacturer's protocol. We obtained 11 ascitic fluid samples (4 ml) from 13 patients. Microbial DNA was extracted using a modified "Godon" protocol. In this regard, after a 10-min centrifuge at 14000 rpm, the pellet was subjected to the same procedure as the faecal samples. However, the final resuspension of the nucleic acids was carried out with 30 µl of a Tris-EDTA buffer solution.

An equivalent of 1 mg of each sample was used for DNA quantification using a NanoDrop ND-1000 Spectrophotometer (Nucliber©). DNA integrity was examined by micro-capillary

electrophoresis using an Agilent 2100 Bioanalyzer with the DNA 12,000 kit, which resolves the distribution of double-stranded DNA fragments up to 17,000 bp in length.

For profiling microbiome composition, the hyper-variable region (V4) of the bacterial and archaeal 16S rRNA gene was amplified by PCR. On the basis of our analysis done with Primer Prospector software, the V4 primer pairs used in this study were expected to amplify almost 100% of the bacterial and archaeal domains. The 5' ends of the forward (V4F_515_19: 5'-GTGCCAGCMGCCGCGGTAA-3') and reverse (V4R_806_20: 5' -GGACTACHVGGGTWTCTAAT-3') primers targeting the 16S gene were tagged with specific sequences as follows: 5'-{AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGT}[3,8,11] {GTGCCAGCMGCCGCGGTAA}-3' and 5'-{CAAGCAGAAGACGGCATACGAGAT} {Golay barcode} {AGTCAGTCAGCC} {GGACTACHVGGGTWTCTAAT}-3'. Multiplex identifiers, known as Golay codes, had 12 bases and were specified downstream of the reverse primer sequence (V4R_806_20).[12,13]

Standard PCR using 0.75 units of Taq polymerase (Roche) and 20 pmol/µ L of the forward and reverse primers was run in a Mastercycler gradient (Eppendorf) at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 56°C for 60 sec, 72°C for 90 sec, and a final cycle of 72°C for 10 min. Amplicons were first purified using the QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain), quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber©) and using an Agilent 2100 Bioanalyzer with the DNA 1000 kit, and then pooled in equal concentration. The pooled amplicons (2 nM) were then subjected to sequencing using Illumina MiSeq technology at the technical support unit of the Autonomous University of Barcelona (UAB, Spain), following standard Illumina platform protocols.

Sequence analysis

Sequences obtained from stool, ascitic fluid, and serum, together with negative controls from the extraction and PCR methods, were analysed with QIIME 1.8.0[14] using an in-house script. Raw sequences of low quality were filtered out with a minimum acceptable Phred score of 20. A demultiplexing step was performed to assign back each read to its corresponding sample and to remove barcodes. A total of 3,393,253 high quality sequences were finally recovered (2,910,686 for faeces and 482,567 for serum and asctic fluid samples). UCLUST algorithm based on 97% of similarity was used to cluster similar sequences into Operational Taxonomic Units (OTUs) or taxa. Representative sequences of each OTU were aligned using PyNAST against Greengenes template alignment (gg_13_8). Chimeric sequences were then identified and

removed with ChimeraSlayer. Finally, a taxonomical assignment for each OTU was performed with the basic local alignment search tool (BLAST) and the combination of two microbial databases (Greengenes and PATRIC). A phylogenetic tree was obtained with the FastTree program. The general OTU table was split into various tables in order to individually analyse faeces, serum, and ascitic fluid samples.

In order to avoid false positive OTUs in stool samples, we eliminated those that did not represent at least 0.2% of the sequences. For samples with a low biomass, such as serum and ascitic fluid, we removed the OTUs that did not account for at least 0.2% of the sequences in at least 3 samples. Moreover, OTUs detected in negative controls were also removed for downstream analyses. Unknown bacteria assigned by BLAST against Greengenes and PATRIC databases were additionally checked against the NCBI database, and OTUs identified as from human origin were removed from the dataset. The final total, mean, minimum and maximum number of sequences per sample type were computed, and OTU tables were rarefied at several rarefaction depths (**Annex 3, Supplementary Table 1**).

Statistical analyses

The characteristics of patients with and without ascites were compared using Fisher's exact test for categorical variables and Mann-Whitney test for quantitative variables. For sequence analysis, pairwise comparisons were performed using OTU tables generated from each sample type. Samples that contained fewer reads than the rarefaction depth were removed for the alpha and beta diversity analyses. The Shapiro-Wilk test was used to check normality of the data, and pairwise comparisons were made between the study groups with the non-parametric test Kruskal-Wallis one-way analysis of variance, which compares means between groups. FDR corrected p-values were taken into account to consider significant results. Richness provided by alpha diversity was computed with Chao1 index. Sample clustering was performed using UPGMA and PCoA methods based on UniFrac metrics.

RESULTS

Enrolment process

A total of 60 outpatients with cirrhosis were evaluated. Thirty-three were excluded due to treatment with non-absorbable disaccharides and/or antibiotics (n = 11), current alcohol intake (n = 7), hepatocellular carcinoma (n = 5), spontaneous bacterial peritonitis (n = 1), other infections or suspicion of infection (n = 3), severe comorbidities (n = 4), or because they were unwilling to participate in the study (n = 2). Therefore, a total of 27 patients were included—13 with ascites and 14 without ascites. Seventeen healthy controls were included for stool (n = 17) and serum (n = 7) microbiome analysis.

Patient characteristics

The characteristics of patients are shown in **Annex 3, Supplementary Table 2**. The main differences between the two groups of patients consisted, as expected, of a more advanced liver insufficiency as determined by the Child-Pugh score and a higher incidence of previous ascites in patients with than in those without ascites. When analysing other factors that could influence the microbiome composition, we did not find statistical differences between the two groups regarding age, body mass index or aetiology of cirrhosis. Patients without ascites showed a trend towards a lower prevalence of diabetes than those with ascites and they were more frequently receiving treatment with beta-blockers or proton pump inhibitors. These differences, however, did not reach statistical significance.

No patient in either group presented symptoms, signs at physical examination or analytical data suggesting infection. Microbial cultures were negative, and neutrophil count was $<250/mm^3$ in all ascitic fluid samples. Therefore, all patients with ascites were considered to have a non-infected ascitic fluid.

Microbiome in stool

The stool microbiome of 27 patients with cirrhosis was compared to that of 17 healthy controls. Alpha-diversity analysis showed that the faecal microbial community of healthy controls presented a higher diversity than that of patients with cirrhosis (**Figure 1a**). However, the diversity was similar in patients with or without ascites (**Figure 1b**). Together, these results

suggest that a loss of microbial diversity in faecal samples is associated with cirrhosis without ascites, but the progression to ascites is not associated with a further loss of diversity.

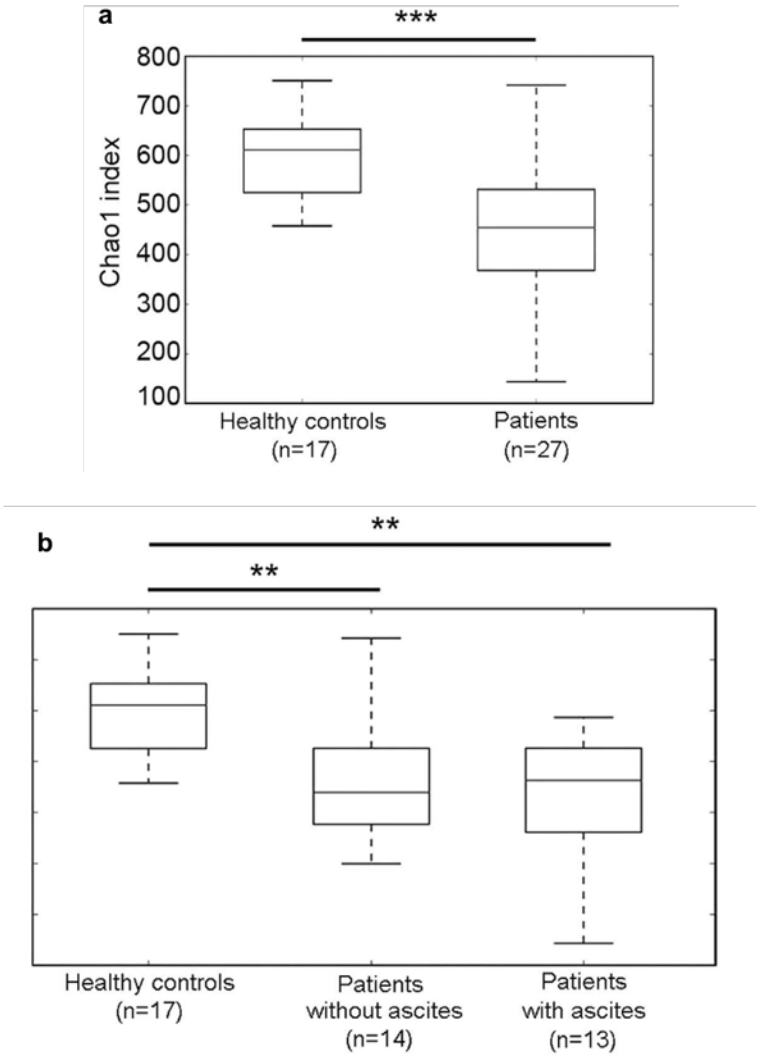


Figure 1. Faecal microbiome of cirrhotic patients and healthy controls. (**a, b**) Healthy controls presented higher microbial diversity compared to all cirrhotic patients (**a**) and to patients with and patients without ascitic fluid (**b**) as assessed by the Chao1 index. The two groups of patients with and without ascites were not significantly different. Analyses were performed on 16 S rRNA V4 region data, obtained from stool samples, rarefied to a depth of 19,930 reads per sample. Healthy controls (n = 17); patients (n = 27); patients with ascites (n = 13); patients without ascites (n = 14); ***P = 0.001; **P = 0.003.

Clustering analysis using PCoA and UPGMA methods based on UniFrac metrics showed that the stool microbiome of cirrhotic patients clustered separately from that of healthy controls (**Figure 2a, b**).

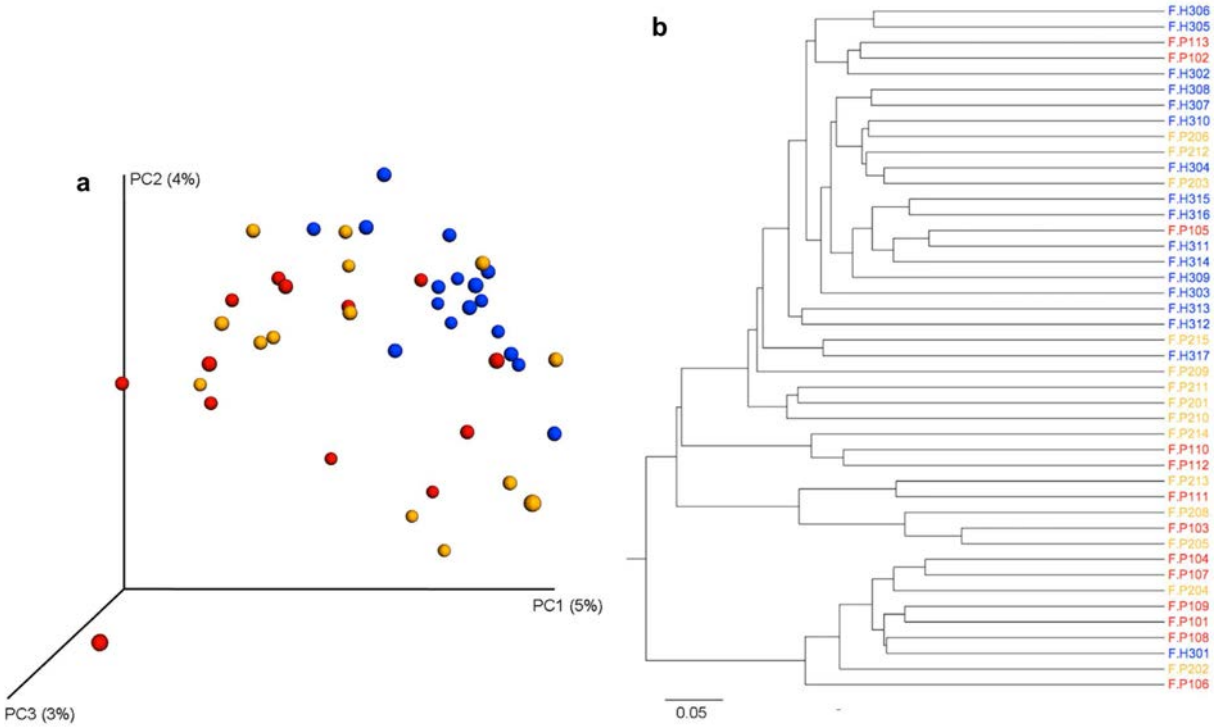


Figure 2. Faecal microbiome of cirrhotic patients and healthy controls. (**a, b**) Unweighted UniFrac PcoA (**a**) and weighted UniFrac UPGMA (**b**) clustering analysis. Blue: healthy controls; orange: patients without ascites; and red: patients with ascites.

At the taxonomic level, patients with cirrhosis were depleted of six species (FDR < 0.05; Kruskal-Wallis test): unknown Clostridiales, *Roseburia faecis*, *Alistipes putredinis*, unknown *Oscillospira*, unknown Mogibacteriaceae, and unknown *Dehalobacterium*, but were enriched in an unknown Peptostreptococcaceae compared to healthy controls (FDR < 0.05; Kruskal-Wallis test; **Figure 3a**. Proteobacteria, at the phylum level, were more abundant in cirrhotic patients than in healthy controls but the difference did not reach significance (FDR = 0.42; Kruskal-Wallis test). All together, these results confirm previous findings that the microbiome composition of cirrhotic patients is altered[4].

Cirrhosis can progress to ascites, which is defined as the accumulation of fluid in the peritoneal cavity. Interestingly, when we analysed the stool microbiome of patients with ascites and those without ascites separately, only the former displayed a significant dysbiosis at the species level, with depletion of unknown Ruminococcaceae, Clostridiales and Peptostroptococcaceae, *Roseburia faecis* and *Alistipes putredinis* and with an enrichment of

Veillonella dispar compared to healthy controls (FDR < 0.05; Kruskal-Wallis test; **Figure 3b**). For several of these species, such as *Roseburia faecis*, *Alistipes putredinis* and *Veillonella dispar,* our findings are in line with those of Qin *et al*.[4] and further support the notion that the progression of the disease is associated with a greater dysbiosis, as reported by Bajaj *et al*.[5]. Patients without ascites presented only a trend towards lower relative abundance of unknown Mogibacteriaceae and *Alistipes* (FDR = 0.053; Kruskal-Wallis test) compared to healthy controls.

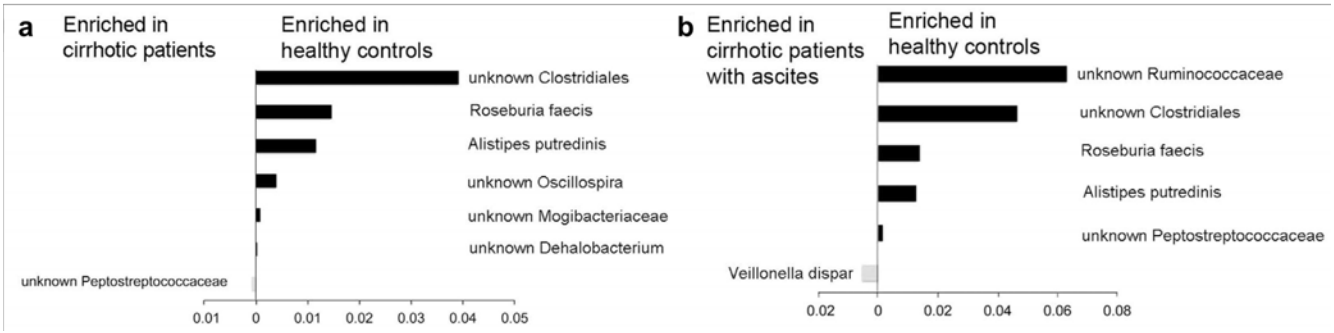


Figure 3. Faecal microbiome of cirrhotic patients and healthy controls (**a, b**) Relative abundance of microbes differentially present at the species level between healthy controls and all cirrhotic patients (**a**) and between healthy controls and cirrhotic patients with ascites (**b**) (Kruskal-Wallis; FDR < 0.05).

Microbiome in fluids

Standard diagnostic microbiological analysis revealed that the serum and ascitic fluid samples were negative for bacterial growth. We analysed the microbiome serum from 7 healthy controls and from the 27 patients and ascitic fluid from 11 patients. Analysis of the 16S rRNA gene of such low-biomass samples may generate contamination at various steps of the process. Therefore, we applied strict protocols for sample collection, DNA extraction, and PCR amplification. For sample collection, we used gloves and proceeded in sterile conditions. For DNA extraction, we used chemicals such as DNA terminator (Biotools, B & M Labs, Spain) to degrade any trace of contaminant DNA in laboratory equipment, and we added negative controls (blanks) during extraction. During PCR amplification, we used UV to clean consumables and H2O and also added PCR blanks.

The amplicons were analysed in an electrophoretic gel and their presence was indicated by a DNA band at about 400 bp (**Annex 3, Supplementary Figure 1**). No DNA band was observed for four control serum samples out of seven, one serum sample from patient with ascites and

one ascitic fluid sample, or for the negative controls added during the extraction (NEG1 and NEG2) and PCR (NEG3) procedures. The PCR amplifications of serum and ascitic fluid samples provided a gradient of intensity in the DNA bands, as analysed in the electrophoretic gel (**Annex 3, Supplementary Figure 1**), in the following order: healthy control serum < cirrhotic patients without ascites < cirrhotic patients with ascites < ascitic fluid, thereby also suggesting a gradient in the microbial load. To remove potential false positive OTUs during sequence analysis, we subtracted sequences with abundant taxa generated in the blanks from the serum and ascitic fluid samples and applied a more restricted filter to the data obtained from samples in order to remove taxa with a low abundance, as specified in the method section. The contamination present in the negative controls was identified as being mostly Proteobacteria (69%) at the phylum level and *Pseudomonas* (30%), *Halomonas* (18%) and unknown (12%) at the genus level. After this filtering step and at a rarefaction of 1000 sequences per sample, we obtained sequence data for 24 out of 27 serum samples from patients and for eight out of 11 ascitic fluid samples and no sequence data were recovered from healthy controls. **Supplementary Figure 2** (**Annex 3**) shows the taxonomic profiling of the three sample types at the phylum level before and after the sequence-filtering step.

Beta-diversity analysis, which studies the variation in composition between samples, showed a similar microbial composition between serum and ascitic fluid samples. However, the microbial community differed greatly between these two sample types and the stools (**Figure 4**), although 89% and 86% of the serum and ascitic fluid microbiome was shared with the stool microbiome at the genus level (**Annex 3, Supplementary Figure 3**).

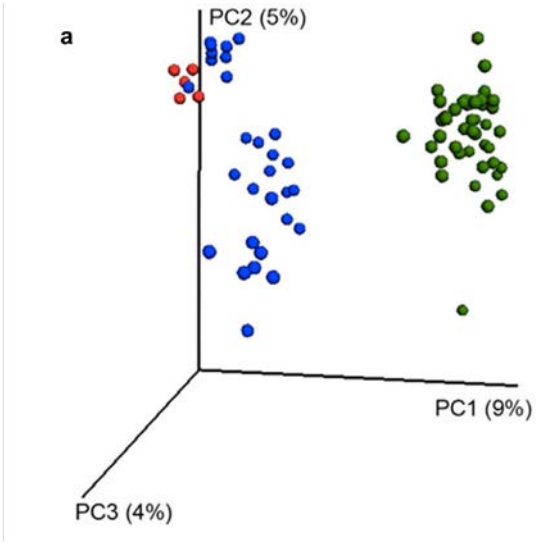
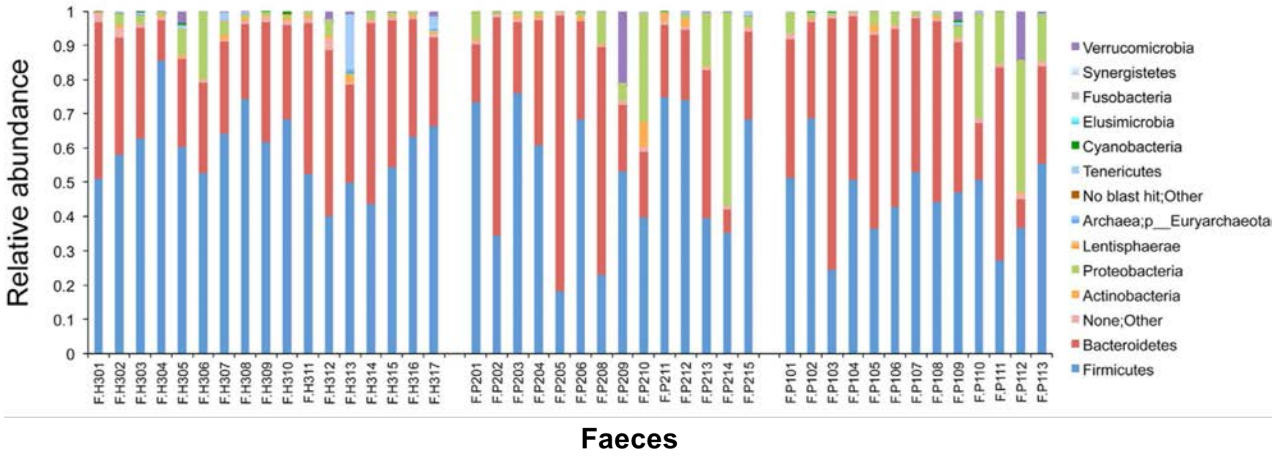


Figure 4. Faecal, serum and ascitic fluid microbiome. Clustering of samples using unweighted UniFrac PcoA representation. Analyses were performed on 16 S rRNA V4 region data, rarefied to a depth of 19,930 reads for stool and 1,000 reads for serum and ascitic fluid samples. Green: stool; blue: serum; red: ascitic fluid.

Euryarchaeota (phylum level) was detected only in stool samples and Thermi and Deinococcus-Thermus were detected only in ascitic fluid (**Figure 5**). Firmicutes and Bacteroidetes were the two most dominant phyla in the three sample types.



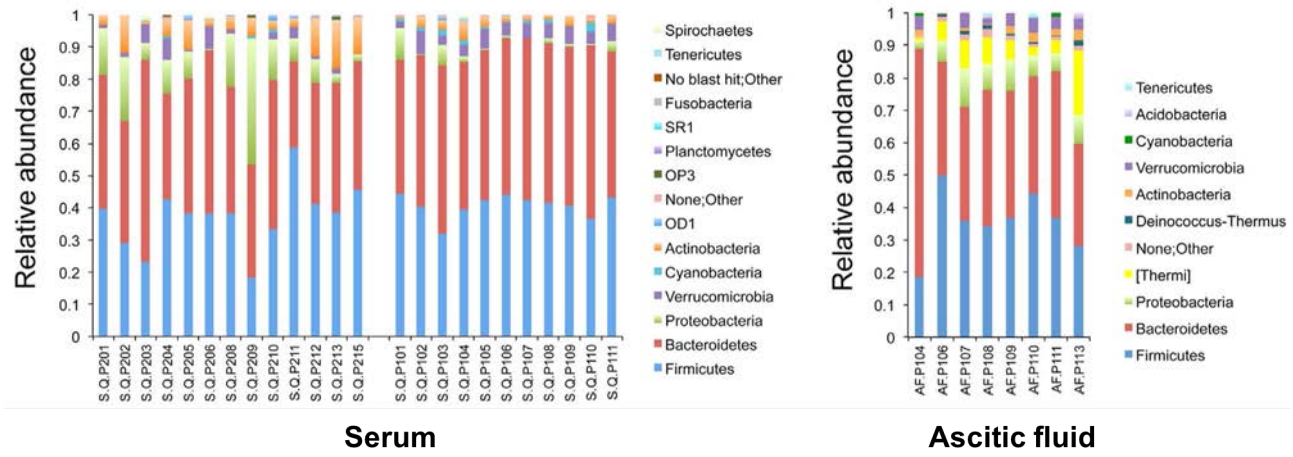


Figure 5. Faecal, serum and ascitic fluid microbiome. Taxonomic composition at the phylum level of the three sample types: Faeces, serum, and ascitic fluid. F.H = Faeces of healthy controls; F.P = Faeces of patients with cirrhosis; S.Q.P = Serum of patients; AF.P = ascitic fluid of patients. 201 to 215 = patients without ascites; 101 to 113 = patients with ascites.

From serum and ascitic fluid, we detected six and eight groups of microbes at the phylum level, 26 and 28 groups at the family level, and 36 and 38 groups at the genus level, respectively. At the phylum level, Firmicutes (41%), Bacteroidetes (37%) and Proteobacteria (14%) accounted for 92% of the sequence data of the serum microbiome, whereas in ascitic fluid Firmicutes (46%), Bacteroidetes (27%), Thermi (10%) and Proteobacteria (8%) accounted for 92%. Serum and ascitic fluid were similar in terms of diversity and richness, as assessed by an abundance-based richness estimator (Chao1) (**Figure 6a**). However, serum specimens of patients with ascitic fluid presented a more diverse microbiome (P = 0.008) than those of patients without (**Figure 6b**), and a significantly higher concentration of lipopolysaccharide binding protein (LBP) (P = 0.02, Mann Whitney test), a marker of microbial translocation (**Figure 6c**). This observation could be explained by patients with ascites, who are expected to have a greater deterioration of the intestinal barrier integrity, also having a higher degree of microbial translocation than those without ascites, thus leading to a higher microbial diversity in serum.

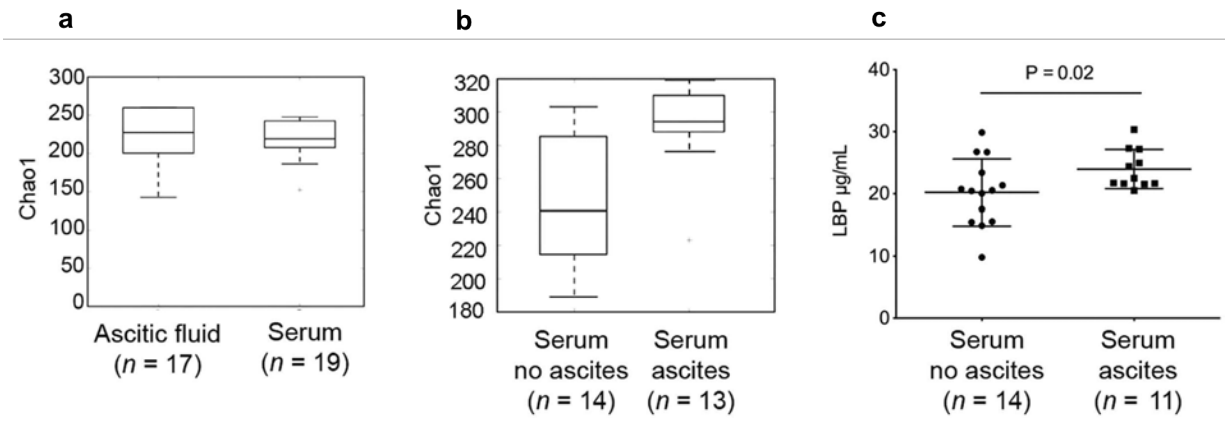


Figure 6. Microbial of extra-intestinal sites and marker of translocation. (**a**) Alpha-diversity of the microbial fluid samples as assessed by Chao1 index of diversity. Ascitic fluid (n = 11); Serum of patients with cirrhosis (n = 19; instead of 27 due to rarefaction depth with ascitic fluid samples). (**b**) Higher alpha-diversity of serum microbiome of cirrhotic patients with ascites compared to that of patients without ($P < 0.05$). Analyses were performed on 16 S rRNA V4 region data, rarefied to a depth of 1,000 reads per sample. (**c**) LBP levels as assessed by specific ELISA; serum of patients with ascites (n = 11 available samples).

Furthermore, using an UPGMA clustering method of the serum microbiome based on an unweighted UniFrac metric, the microbiome of patients with and without ascites clustered separately (**Figure 7a**). This result suggests that a specific serum microbiome is linked to the presence of ascites.

Taxonomic comparison showed that an unknown group of microbes at the family level, belonging to the Clostridiales order, displayed a higher relative abundance in serum of patients with ascites (FDR = 0.03; Kruskal-Wallis test) and another group, Moraxellaceae, showed a lower relative abundance in patients with ascites compared to those without (**Figure 7b**). Interestingly, this group of bacteria was also found in ascitic fluid samples (**Annex 3, Supplementary Figure 4**), thereby supporting the notion of translocation from serum to ascitic fluid. At the genus and species level, an unknown genus related to Cyanobacteria (FDR = 0.002) was found in higher relative abundance in patients with ascites compared to those without.

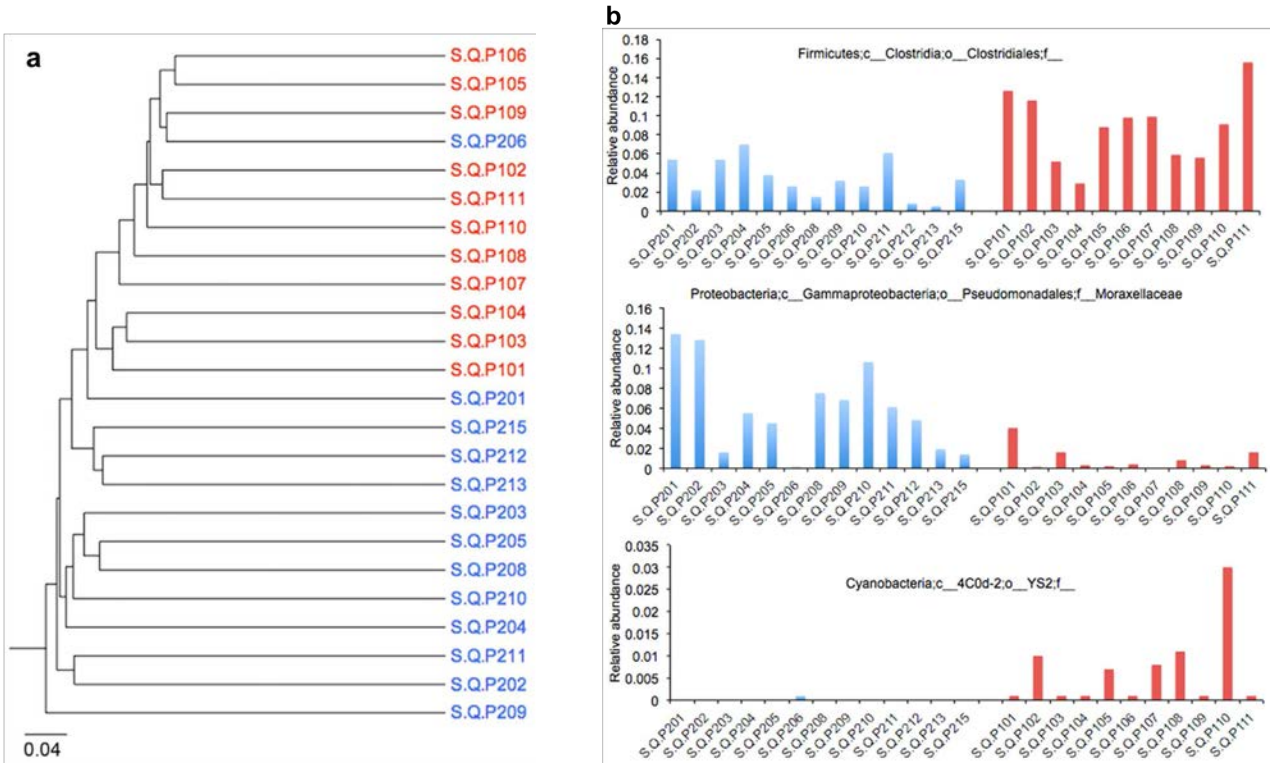


Figure 7. Serum microbiome of patients with and without ascites. (**a**) UPGMA clustering based on unweighted UniFrac metric of serum samples of cirrhotic patients with and without ascites. (**b**) Relative abundance of microbes or groups of microbes significantly different between serum microbiome of cirrhotic patients with and without ascites. Analyses were performed on 16S rRNA V4 region data, rarefied to a depth of 1,000 reads per sample.

Bacterial translocation

In order to study whether the presence of bacterial DNA in ascitic fluid and blood derived from the gastrointestinal tract, we counted the taxa common to stool and serum, stool and ascitic fluid, and serum and ascitic fluid. For this purpose, we first counted the number of taxa in each sample type, finding an average of 397 (SD = 94), 283 (SD = 76) and 97 (SD = 25) taxa in stool, serum and ascitic fluid, respectively. By comparing the taxa between samples, we detected on average 37 taxa common to both stool and serum, 20 to serum and ascitic fluid, and three to ascitic fluid and stool (**Annex 3, Supplementary Figure 5**). These results indicate that the three sites share few common microbial taxa and therefore suggest that the microbial taxa present in the serum, but not detected in stool, could either take root in extra-intestinal sites such as the lung or the vagina for women or were in too low abundance in the stool to be detectable

but when they reached the serum, a more appropriate environment for their growth, they became detectable.

DISCUSSION

This is the first study to validate the presence of polymicrobial DNA in both the serum and ascitic fluid of patients with cirrhosis using high-throughput sequencing techniques. Our findings showed that the microbial community in serum and ascitic fluid, although showing more than 80% similarity with that of the stool microbiome at the genus level, is specific and complex at the taxa level. Previous studies using a variety of techniques, mainly conventional PCR, reported the presence of bacterial DNA in ascitic fluid and/or blood only in up to 30–60% of these patients[15–18]. Moreover, most of the DNA detected in these studies was monomicrobial, identified as being *Escherichia coli* or *Staphylococcus aureus*[15–18]. A recent study has reported the characterization of the microbial composition of the ascetic fluid of cirrhotic patients[19]. However, the authors amplified the 16S gene from only one individual out of seven and this individual was positive for *Escherichia coli* in culture. Using shotgun-sequencing technique on two pools of ascitic fluid obtained from three patients, they were able to identify only 0.1% of bacterial DNA, for which the majority was identified as being *Escherichia*. However, according to our findings, *Escherichia* belonging to the Proteobacteria phylum could also be found in the extraction and PCR blanks. We therefore recommend that future studies on samples with a low biomass include several blanks and minimise the amount of *Taq* polymerase used during the PCR amplification, since it may contain contaminant DNA. The detection of polymicrobial DNA in the serum and ascetic fluid observed in the present study is in line with our previous findings in rats, showing a high microbial diversity in MLNs of a model of CCl_4-induced liver injury, as well as in those of control rats.[7]

We were unable to analyse the serum microbiome of the seven healthy controls at a sufficient rarefaction depth compared to all other samples. Indeed, the presence of DNA bands in the electrophoretic gel after serum amplification could be due to the presence of human DNA combined with contaminant DNA during extraction and amplification, thus impeding analysis of the microbiome of these samples after filtering out the contaminant sequences. As the same method of sample collection and processing was used for patients with cirrhosis, this finding supports that the detection of bacterial DNA in patients with cirrhosis was not caused by contamination. This observation suggests that healthy individuals harbour a very low or undetectable microbial load in blood, which is in agreement with a recent study demonstrating the presence of a gut-vascular barrier that controls the systemic dissemination of bacteria in healthy individuals but not in patients with celiac disease and liver damage[20]. In cirrhotic

patients, the similarity of the microbiome composition between serum and ascitic fluid compared to stool samples could be due, in part, to the body site selecting only microorganisms capable of growing in a liquid and relatively aerobic environment. The differences found in diversity (Chao1 index) and in composition and structure of the serum microbiome between patients with and without ascites are alterations that are associated with cirrhosis progression, thereby validating the assumption of previous studies[21].

The decrease in stool microbial diversity and the depletion of several commensal groups of bacteria (unknown Ruminococcaceae, Clostridiales and Peptostroptococcaceae, *Roseburia faecis* and *Alistipes putredinis*) in patients with cirrhosis is also in agreement with the findings of previous studies[3,4]. However, in contrast to other authors[5], we did not observe a significant increase in potential pathogenic bacteria such as Enterobacteria, but only a trend towards an increase in Proteobacteria or Streptococcaceae. This observation could be explained by a smaller sample size and the fact that the patients in our study presented a relatively preserved liver function, as reflected by the low Child-Pugh and MELD scores, in comparison with other studies that included groups with more advanced liver failure.

Our study presents several limitations such as a small sample size, DNA contamination that may remain after sequence curating (despite the multiple precautions to avoid this as mentioned above), and confounding factors. To reduce possible confounding factors, we excluded patients with recent alcohol intake and those treated with antibiotics or non-absorbable disaccharides. We did not find statistically significant differences between patients with and without ascites in other possible confounding factors, such as diabetes and the use of beta-blockers or proton-pump inhibitors. However, we cannot exclude that the non-significant differences observed in these parameters could have influenced the results reported here.

Despite these limitations, we conclude that serum and ascitic fluid of patients with cirrhosis contain a complex and specific microbial community and that our method of low-biomass analysis could be applied to other conditions of gut-vascular barrier failure[20]. We propose that alteration of the serum and fecal microbiome composition be considered indicators of cirrhosis progression.

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CHAPTER 2

CHAPTER 2. CIRRHOSIS IN AN ANIMAL MODEL (RATS)

"Sequential changes in the mesenteric lymph node microbiome and immune response during cirrhosis induction in rats"

ABSTRACT

Progression of cirrhosis is associated with changes in the gut microbiome. Microbial translocation from the gastrointestinal tract to extra-intestinal sites plays a major role in poor disease outcome and survival in cirrhotic patients. However, whether the interaction between the gut microbiota and the immune response influences the evolution of cirrhosis is poorly understood. We aimed to investigate the modification of the microbiome and the immune response during the progression of CCl_4-induced cirrhosis in rats in a spatial and temporal setting.

The microbiome of MLNs, blood and ascitic fluid (AF) showed distinctive composition and function compared to stool and ileo-cecal content (ICC), thus validating a compartmentalised microbiome in both control and CCl_4-treated rat groups. Upon CCl_4-induction, pathobionts attempted to displace symbionts in ICC samples, and finally symbionts failed to compensate pathobionts when rats developed ascites. Microbial load increased and showed a positive correlation with the relative abundance of pathobionts in the MLN of ascitic (decompensated) rats. Among several genera, *Escherichia* and *Candidatus Arthromitus*, were correlated with elevated levels of systemic pro-inflammatory cytokines, TNF-α and IL-6. *Candidatus Arthromitus*, a well-known segmented filamentous bacterium (SFB), was detected in ICC, MLN and AF samples, suggesting a possible translocation from the gut to the AF through the lymphatic system, whereas Escherichia was detected in ICC, MLN, AF and also blood, suggesting a possible translocation from the gut to the AF through the blood stream.

In the present study, we demonstrate that microbiome changes in intestinal sites are associated with microbial shifts in the MLNs as well as an increase in cytokine production, providing further evidence of the role of the gut-liver-immunity axis in the progression of cirrhosis.

INTRODUCTION

Cirrhosis is defined as the presence of fibrosis and regenerating nodules in the liver due to various causes such as alcohol, hepatitis viruses, metabolic syndrome or immune dysfunction, Cirrhosis can lead to portal hypertension and liver insufficiency, and their related complications, such as infections, hepatic encephalopathy, ascites, hepatorenal syndrome, variceal bleeding and ACLF[1]. Before the development of complications patients are considered to have “compensated cirrhosis”, and when complications develop they are considered as having “decompensated cirrhosis”, which has a poorer prognosis than patients at the compensated stage[2].

Patients with cirrhosis present complex alterations in their cross-talk between the gut microbiome and the immune system that contribute to the development of complications and therefore to the evolution from compensated to decompensated stage[3].

The intestinal microbiota, which harbours 100 trillion bacteria, archaea and eukarya, is known to play a pivotal role in the development of the host immune system and in the maintenance of host homeostasis by suppressing responses to pathogens and by enforcing the integrity of the barrier functions of the gut mucosa[4]. Commensal bacteria are transported by DCs through the lymphatic system from the gut to the MLNs, which forms part of the GALT and acts as the first line of immune defence against pathogens from the intestines[5-7]. In the MLNs bacteria are maintained at low levels by the host mucosal immune system[8].

Patients with cirrhosis present alterations in gut microbiota, intestinal permeability and immune response leading to bacterial translocation, which is the passage of viable bacteria from intestinal lumen through the intestinal wall to the MLNs or other sites[7-10]. Then, the gut-liver-immune axis is activated by BT, which stimulates the induction of pro-inflammatory cytokines further perpetuating increased intestinal permeability and thus BT[11]. PAMPs, such as the endotoxin LPS found on the cell membrane of gram-negative bacteria, bind to PRRs such as toll like receptors (TLRs) causing an induction of pro-inflammatory cytokines like TNF-α and IL-6, which tend to be elevated in patients with cirrhosis[12]. MLNs also produce TNF-α in response to bacterial translocation, especially in patients with ascites[6, 13, 14]. Ascites is a common complication in advanced cirrhosis that is associated with a high mortality rate and is caused by PHT leading to fluid accumulation in the abdomen. It has been hypothesised that elevated TNF-α production causes hemodynamic disturbances leading to splanchnic

vasodilatation through NO synthesis stimulation which could contribute to altered intestinal barrier function resulting in bacterial translocation[6, 15], which has been observed in cirrhotic patients with ascites. Additionally, ascites has been shown to increase the susceptibility of host bacterial infection[11] likely due to the fact that TNF-α has been shown to loosen tight junction proteins of intestinal epithelial cells perpetuating bacterial translocation and subsequent inflammatory response[7].

Recently, Bajaj *et al*. has shown that severity of cirrhosis in patients was associated with progressive changes in the gut microbiome in a longitudinal study[3]. In fact, recent evidence shows that bacterial translocation from the intestines could play a major role in poor disease outcome and patient survival[16, 17]. However, the underlying mechanisms that involve the gut microbiota in the disease progression are not well understood.

Therefore, the aims of this study were to i) investigate the spatial and temporal changes of the composition and function of the microbiome in a cirrhosis rat model; ii) evaluate changes of the microbiome as they relate to the progression of cirrhosis; iii) assess the immune modulation by the microbiome detected in extra-intestinal sites.

METHODS

Experimental design

Animals. Male Sprague-Dawley rats weighing 35–49 g were purchased from Harlan Laboratories (Indianapolis, Ind., USA) and provided by Research Models and Services Production (Udine, Italy). After the rats were weaned from their mothers, they were fed a rodent chow diet (2018S; Teklad, Madison, Wisc., USA). After a 1-week quarantine, all animals were placed in individual cages and kept at a constant room temperature of 21°C, exposed to a 12-hour light:12-hour dark cycle and allowed free access to water and rodent chow (A04; SAFE, Augy, France). One week later, phenobarbital (1.5 mmol/l) (Luminal, Kernpharma, Barcelona, Spain) was added in tap water in all animals. There was no contact between rats neither through water, chow or faeces of other animals.

Induction of cirrhosis and study groups. Cirrhosis was induced as previously described[18]. When rats reached a weight of 200 g, they were administered weekly doses of CCl_4 (Sigma-Aldrich, St. Louis, Mo., USA) intragastrically using a sterile pyrogen-free syringe (ICO plus 3 Novico Médica, S.A., Barcelona, Spain) with an attached stainless-steel animal feeding tube (Popper and Sons, New Hyde Park, N.Y., USA) without anaesthesia. The first dose of CCl_4 was 20 µl, and subsequent doses were adjusted on the basis of changes in weight 48 h after the previous dose. When cirrhotic rats presented ascites, the dose of CCl_4 was maintained at 40 µl.

We designed different groups of cirrhotic rats for which laparotomy and sample collection were performed at four different time points: after 6, 8, 10 weeks of the first dose of CCl_4, and the last group when ascites was suspected by the increase in abdominal girth and confirmed by paracentesis. A control group with non-cirrhotic rats was also included and samples were collected at the same four time points that the cirrhotic group. Paracentesis was performed under air anesthesia with isofluorane (Forane ®, Abbott, Madrid, Spain) in sterile conditions, and approximately 0.1 ml of ascitic fluid was removed. One week later, a laparotomy was carried out.

Laparotomy. Laparotomy was performed in all cirrhotic and control rats at weeks 6, 8, 10 or when ascites was suspected. For laparotomy, rats were anesthetised with 10 mg/kg xylazine (Rompun ®; Bayer, Kiel, Germany) and 50 mg/kg ketamine (Ketolar®; Parke-Dawis, Madrid, Spain) in sterile conditions. In brief, the abdominal fur was removed with a depilatory cream

and the skin was sterilised with iodine (Curadona, Lainco, Spain). The abdomen was then opened via a 4-cm median incision, and the remaining fluid was removed.

Biological sample collection

The sequence of sample collection at laparotomy was: stool (before laparotomy), MLN, blood, liver spleen, ICC. Samples from CCl_4-treated and control rats were stores frozen at -80°C until microbiome analysis. Blood and MLN samples were also used for cytokine analysis.

Cytokine measurement

TNF-α, IL-6 and IL-10 cytokines were determined in blood samples and IL-17 in MLNs by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocols (eBiosciences). Results are expressed as pg/ml in blood samples and the ratio IL-17 pg per mg of total protein. Limits of detection were 30 pg/ml for TNF-α, IL-6 and IL-17 and 15 pg/ml for IL-10.

Microbiome analysis

Genomic DNA extraction. All biological specimens were processed for genomic DNA extraction using protocols previously used in Santiago *et al*.[19] for low biomass samples such as MLN, blood and AF and the one recommended by the International Human Microbiome Standard[20] for stool samples.

16S rDNA gene sequencing. To prepare the DNA for sequencing, we amplified a fragment of the 16S rDNA gene by PCR using universal primers targeting the V4 hypervariable region as previously described[21]. Amplicons were then purified using the QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain), quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber©) and then pooled in equal concentration. The pooled amplicons (2 nM) were then subjected to sequencing using Illumina MiSeq technology at the technical support unit of the Autonomous University of Barcelona (UAB, Spain), following standard Illumina platform protocols.

Microbiome composition analysis. To analyse the microbiome composition, we first loaded the raw sequences into the QIIME 1.9.1 pipeline, as described by Molina *et al*.[22] Low quality sequence reads were filtered out by applying default settings and a minimum Phred score of 20. From the filtering step, from a total of 214 samples, we obtained a total of 2.5 millions of high-quality sequences with an average number of reads of 11899. We used the USEARCH algorithm to cluster similar filtered sequences into OTUs based on a 97% similarity threshold. We then identified and removed chimeric sequences using UCHIME. Representative sequences were selected and aligned using PyNAST against Greengenes template alignment (gg_13_8 release), and a taxonomical assignment step was performed using the basic local alignment search tool to map each representative sequence against a combined database encompassing the Greengenes and PATRIC databases.

For β diversity analysis, we rarefied to 1046 sequences per sample when comparing all samples simultaneously. When analysing only low biomass samples we rarefied them at 1046 sequences per sample, and at 9396 sequences per sample when analysing stool and ICC samples. Rarefaction is used to overcome cases in which read counts are not similar in numbers between samples. Weighted and unweighted UniFrac metrics were applied to build phylogenetic distance matrices, which were then used to construct hierarchical cluster trees using UPGMA and PcoA representations. To perform α diversity estimates, we calculated the Chao1 and Shannon diversity indexes.

Gene and pathway abundance analysis. PICRUSt version 0.9.1[23] was used to predict the abundance of KEGG orthologous groups (KOs).

Microbial load assessment. To quantify microorganisms, the extracted genomic DNA was used to amplify the V4 region of the 16S rRNA gene by quantitative real-time PCR (qPCR) using the following primers: V4F_517_17 (5'- GCCAGCAGCCGCGGTAA-3') and V4R_805_19 (5'-GACTACCAGGGTATCTAAT-3'). The qPCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems) using optical-grade 96-well plates. The PCR reaction was performed in a total volume of 25 µl using the Power SYBR Green PCR Master Mix (Applied Biosystems), containing 100 nM of each of the universal forward and reverse primers. The reaction conditions for amplification of DNA were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate and mean values were calculated. This experiment was also duplicated to ensure accuracy. Mean values

of both experiments were taken into account. Data were analysed using Sequence Detection Software version 1.4, supplied by Applied Biosystems.

Statistical analysis

We performed statistical analyses under QIIME and R. We used the Agostino_Pearson test to check for normality of data distribution. Parametric normally distributed data were compared by Student's t-test for paired or unpaired data; otherwise, the Wilcoxon signed rank test was used for paired data and the Mann-Whitney U test for unpaired data. The Kruskal-Wallis one-way test of variance was used to compare the mean number of sequences of different unpaired groups of subjects at various taxonomic levels, the Wilcoxon test was used when comparing only 2 groups. We performed analyses with the nonparametric multivariate ANOVA (PERMANOVA) called the Adonis test, a non-parametric analysis of variance, to test for differences in microbial communities. We performed Spearman test to evaluate correlations between microbiome composition and biological parameters such as cytokine levels. When possible, the analysis provided FDR (q)-corrected p values (q values). $q < 0.05$ considered significant for all tests.

RESULTS

We studied 15 control rats at weeks 6 (n = 5), 8 (n = 5) and 10 (n = 5), 25 CCl4-treated rats sacrificed at weeks 6 (n = 9), 8 (n = 8) and 10 (n = 8) that were considered rats with compensated cirrhosis, and 19 CCl4-treated rats when they developed ascites that were considered rats with decompensated cirrhosis.

Compartmentalisation of microbial communities

To confirm the existence of a compartmentalised microbiome, we compared the microbiome composition and function of various body sites: intestinal sites such as faeces and ICC, and extra-intestinal sites such as MLN, blood and ascitic fluid (AF). Faeces and ICC specimens from control rats presented similar microbial communities (p = 0.44; Adonis test; **Figure 1a, b**) dominated by Firmicutes (61% and 78%, respectively), Bacteroidetes (35% and 20%, respectively), Actinobacteria (1% and 0.04%, respectively) and Proteobacteria (0.3% and 0.4%, respectively).

The microbiome of MLN, blood and AF rats showed distinctive composition compared to faeces and ICC (p < 0.0001; Adonis test) (**Figure 1a**). MLNs and blood displayed similar microbial communities (p = 0.616, Adonis test) dominated by Firmicutes (48% and 50%, respectively), Bacteroidetes (43% and 37%, respectively), Proteobacteria (3% and 2%, respectively) and Actinobacteria (3% and 5%, respectively) (**Figure 1b**).

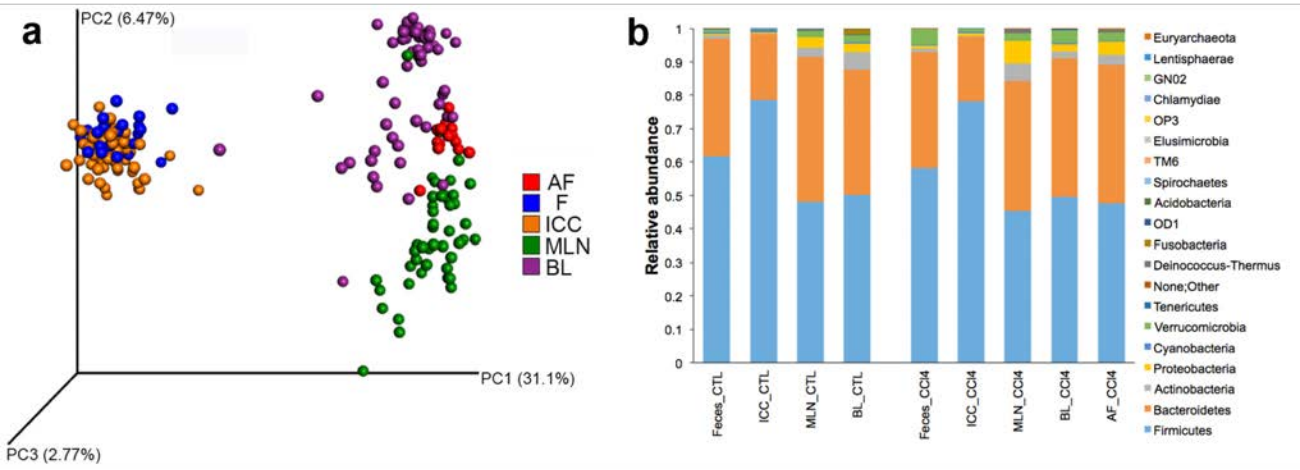

Figure 1. Spatial microbial community compositions. **(a)** Weighted principal coordinate analysis (PCoA) UniFrac metrics (taxonomic clustering). **(b)** Relative abundance of the phyla in the control group (CTL) and

CCl4 treated rats (CCl4). Significant differences ($p < 0.0001$; Adonis test) were observed between extra-intestinal sample sites (blood, mesenteric lymph node (MLN), ascitic fluid (AF)) and intestinal sites (ileo-cecal content (ICC) and stool) for taxonomic clustering plots. n = 23 for stools; n = 59 for ICC; n = 46 for MLN; n = 57 for blood and n = 15 for AF.

To predict microbial functions using 16S rDNA sequences, we used PICRUSt, a bioinformatics software package that uses a full genome database. The analyses revealed that faeces and ICC, compared to MLNs, blood and AF, contained different microbiome functions (**Figure 2a, b**) such as those involved in metabolism, genetic information processing and environmental information processing ($q < 0.003$; Wilcoxon test) that represented more than 80% of the functions (**Figure 2b**). Microbial functions were also similar between ICC and faeces and between MLNs and blood ($q > 0.1$; Wilcoxon test).

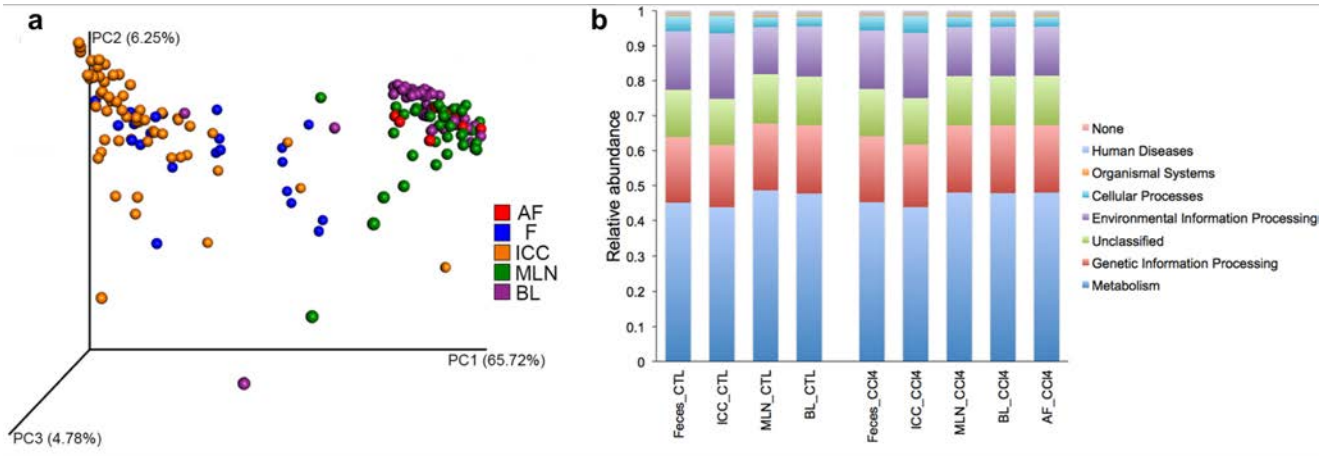

Figure 2. Spatial microbial community functions. (a) Principal component analysis (PCA) plot comparing KEGG module predictions with PICRUSt (functional clustering) using 16S data. **(b)** Relative abundance of the functions in the control group (CTL) and CCl4 treated rats (CCl_4). Significant differences ($p < 0.0001$; Adonis test) were observed between extra-intestinal sample sites (blood, mesenteric lymph node (MLN), ascitic fluid (AF)) and intestinal sites (ileo-cecal content (ICC) and stool) for functional clustering plots. n = 23 for stools; n = 59 for ICC; n = 46 for MLN; n = 57 for blood and n = 15 for AF.

Longitudinal study: Evolution of cirrhosis and microbiome modification

To evaluate the evolution of the microbiome in parallel with the progression of cirrhosis, rats were sacrificed to collect ICC, MLNs and blood at different time points (6, 8 and 10 weeks after

the initiation of CCl_4 treatment and at ascites development for the cirrhosis group and with matched time points for the control group). We found significant changes in the microbiome composition in ICC and MLN samples that were associated with the progression of cirrhosis (**Annex 4, Supplementary Figure 1**)

In their ICC samples, CCl_4-induced cirrhosis rats showed an increase of Betaproteobacteria ($p = 0.01$; $q = 0.098$) and Erysipelotrichia ($p = 0.001$; $q = 0.012$; Kruskal-Wallis test) at weeks 6 and 8 of CCl_4 treatment in comparison to control rats (**Figure 3**). At week 10, both groups of bacteria significantly decreased compared to weeks 6 and 8. When rats developed ascites, Erysipelotrichia almost disappeared whereas Betaproteobacteria increased again. Both groups of bacteria were absent from control rats.

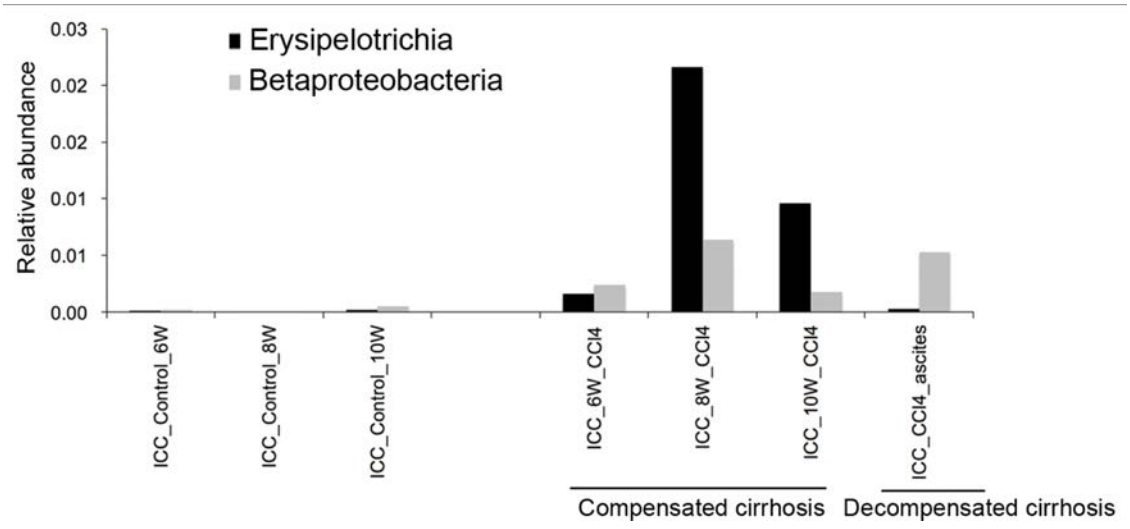

Figure 3. Microbial groups in ICC samples involved in the severity of cirrhosis. Temporal taxonomic difference between controls and CCl_4-treated rats. Two classes of bacteria, Erysipelotrichia ($p = 0.001$; $q = 0.012$) and Betaproteobacteria ($p = 0.01$; $q = 0.098$), presented significantly different relative abundance over time between the control and the CC_4 treated groups. Statistics were performed using the Kruskal-Wallis test.

At the genus level, *Sutterella* and *Coprococcus* increased ($p < 0.001$; $q < 0.02$; Kruskal-Wallis test) in decompensated rats compared to controls while *Desulfovibrio* and *Ruminococcus* increased ($p < 0.002$; $q < 0.025$; Kruskal-Wallis test) in controls (**Figure 4**). At 6, 8 and 10 weeks after CCl_4 treatment, those rats without ascitis presented an intermediate relative abundance of all these genera. The *Allobaculum* genus, known as a potentially beneficial bacterial group,[24] showed an increase in the compensated CCl_4-treated rats but disappeared when they

presented ascites (p = 0.0004; q = 0.013; Kruskal-Wallis test). These results suggest that groups of beneficial bacteria attempted to displace pathogenic ones. This compensation seemed to fail when rats presented ascites. *Candidatus Arthromitus*, a genus from the Firmicutes phylum, showed higher relative abundance only in decompensated rats compared to compensated and control rats (p = 0.023; q = 0.15; Kruskal-Wallis test).

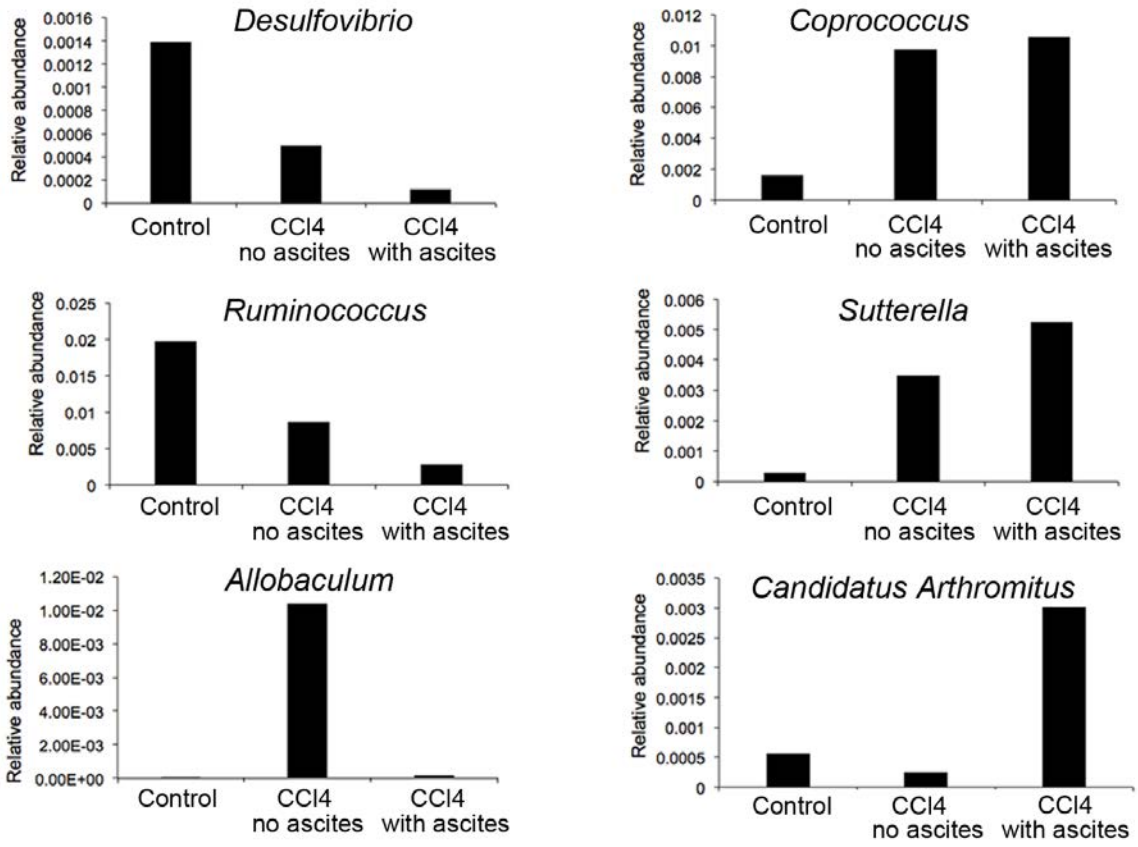

Figure 4. Microbial groups in ICC samples involved in the severity of cirrhosis (genus level). Taxonomic differences between controls and CCl_4-treated rats with ascites and CCl_4-treated rats without development of ascites. Two bacterial genera, *Coprococcus* (p = 0.0001; q = 0.011) and *Sutterella* (p = 0.0005; q = 0.014), were found in higher relative abundances in CCl_4-induced cirrhotic rats than in control rats. Two bacterial genera, *Desulfovibrio* (p = 0.002; q = 0.025) and *Ruminococcus* (p = 0.0007; q = 0.013), were found in higher relative abundance in control rats than in CCl_4-induced cirrhotic rats. *Allobaculum* was found in higher relative abundance (p = 0.0004; q = 0.013) only in compensated cirrhotic rats, and *Candidatus Arthromitus* (p = 0.023; q = 0.15) was in higher relative abundance in decompensated cirrhotic rats. Statistics were performed using the Kruskal-Wallis test.

In MLN samples of decompensated rats (with ascites), only one bacterial genus, *Candidatus Arthromitus*, showed a significantly higher relative abundance compared to control and compensated rats (p = 0.0002; q = 0.019; Kruskal-Wallis test) (**Figure 5**).

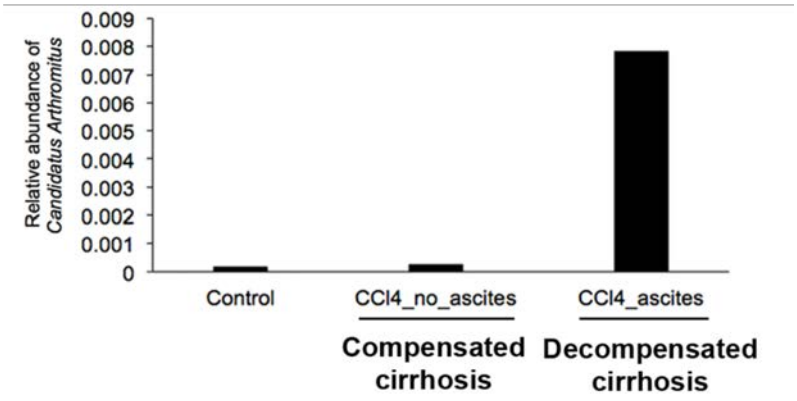

Figure 5. Comparison of relative abundance of *Candidatus Arthromitus* between groups. *Candidatus Arthromitus* was found in higher relative abundance in MLN of decompensated rats (p = 0.0002; q = 0.018).

Candidatus Arthromitus was found in 72% (13 out of 18) of MLN samples of decompensated rats. Moreover, it was identified in 42% (8 out of 19) of the ICC samples, 26% (4 out of 15) of the AF samples, in only 5% of blood samples (1 out of 18) and was not found in stool samples (**Figure 6**).

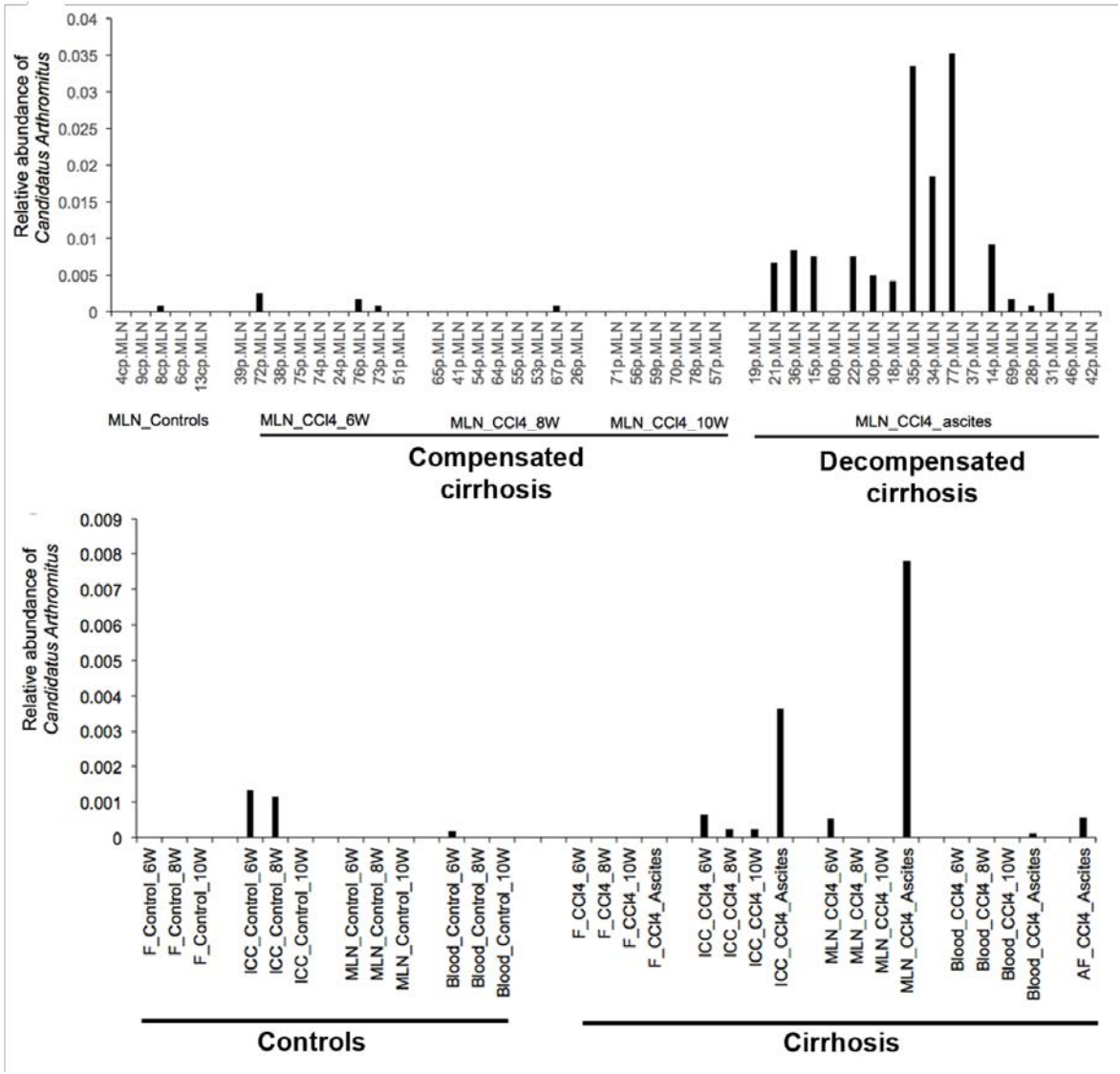

Figure 6. Detection of *Candidatus Arthromitus* in spatial and temporal settings. Detection of *Candidatus Arthromitus* was found in 72% (13 out of 18) of MLN samples of decompensated rats. *Candidatus Arthromitus* was detected in ICC, MLN, blood and AF samples but not in faeces

Prediction analysis showed that functions involved in lipid metabolism (p = 0.0001; q = 0.0269; Wilcoxon test) and in targeting the immune system such as those involved in systemic lupus erythematosus (p = 0.0018; q = 0.19; Wilcoxon test) were enriched in the MLN samples in decompensated rats compared to control rats (**Figure 7**).

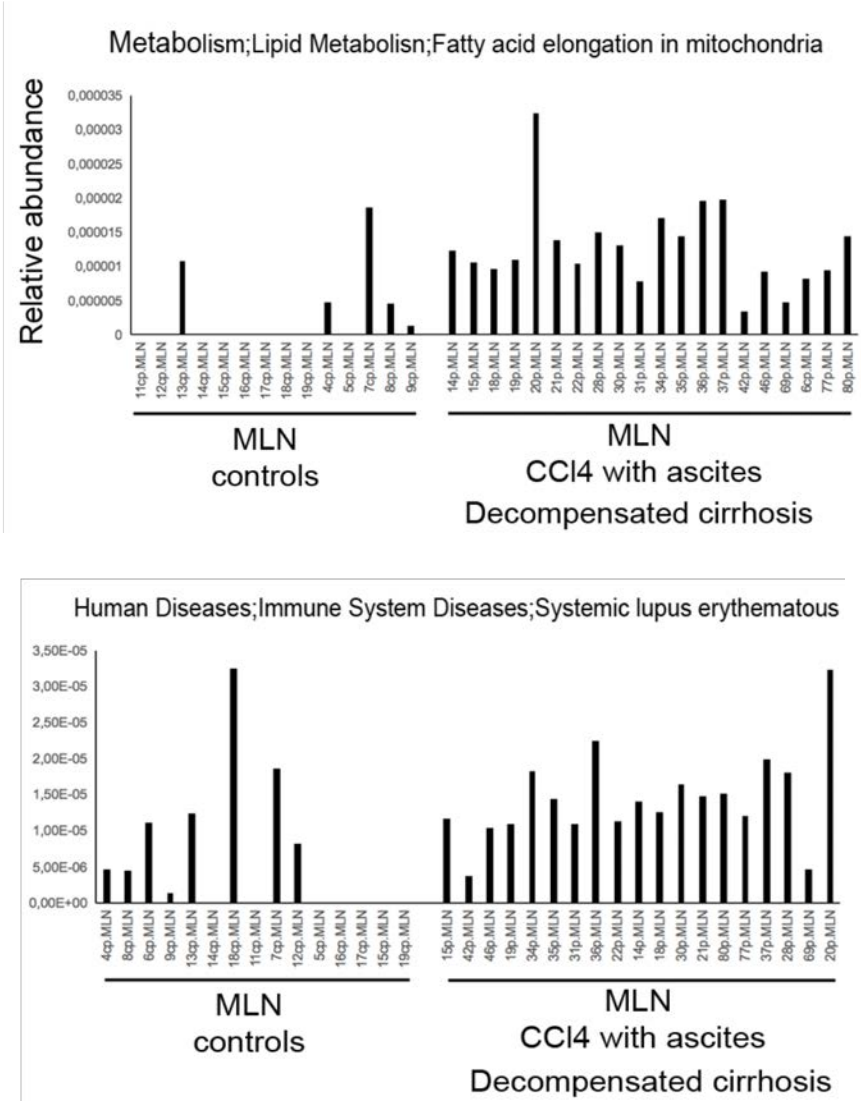

Figure 7. KEGG module predictions using 16S data with PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). Two modules, involved in lipid metabolism (p = 0.0001; q = 0.02) and immune system diseases (p = 0.0018; q = 0.199), were found in higher relative abundance in decompensated rats compared to control rats. Statistics were performed using the Wilcoxon test.

In faeces, *Coprococcus, Sutterella* and *Allobaculum* showed some differences in their relative abundance between the three groups of rats (controls, compensated and decompensated) but none of the differences were significant (q > 0.2; Kruskal-Wallis test). In blood samples, the Spirochaetes phylum was found in higher proportion in the decompensated group compared to the two other groups (p = 0.001; q = 0.035; Kruskal-Wallis test).

Using the weighted UniFrac distance, a metric used to compare microbial community composition between samples, we evaluated the stability of the microbiome in ICC, MLN and blood samples of rats under induction of cirrhosis without ascites compared to rats with ascites. We observed a lower stability of the microbiome composition of rats with ascites in blood samples (p = 0.04; Mann Whitney test) (**Annex 4, Supplementary Figure 2**), but the difference was not significant in ICC and MLN samples.

Microbial load

To validate our findings on relative abundance of the sequenced 16S rDNA gene, we evaluated the microbial load using real-time quantitative PCR of the 16S rDNA gene. Microbial load, as measured by 16S rDNA-qPCR, was significantly higher in the MLN of cirrhotic decompensated rats compared to control rats (p = 0.008; Mann Whitney test), was positively correlated with the relative sequence abundance of Proteobacteria (rho = 0.673; p = 0.002; Spearman test) and was negatively correlated with the relative abundance Bacteroidetes (rho = -0.637; p = 0.004; Spearman test) (**Figure 8**).

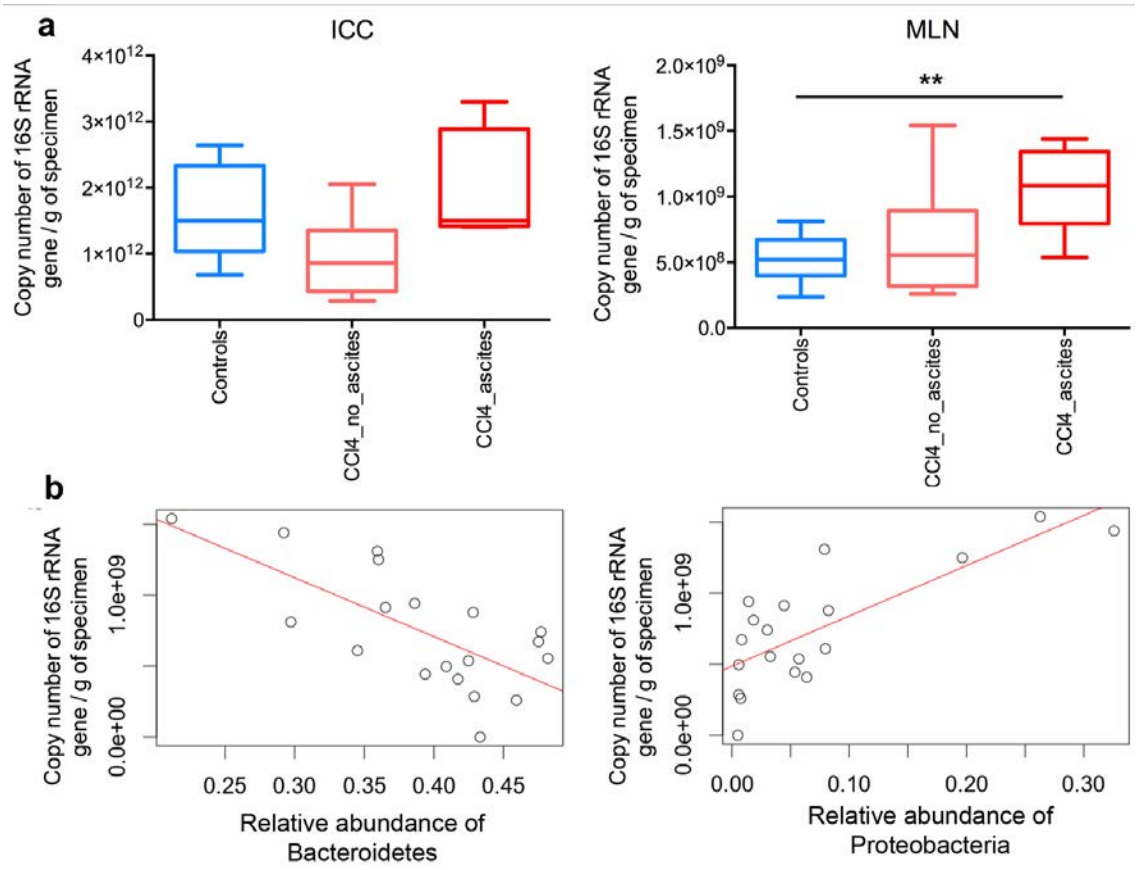

Figure 8. Quantification of the microbiota by real-time PCR on the 16S rRNA gene and correlation with microbiome composition. (a) Microbial load in ICC samples appeared to be higher in decompensated rats compared to control and compensated rats and was found significantly higher in the MLNs of decompensated rats compared to control rats (p = 0.008; Mann Whitney test). In both ICCs and MLNs, n = 8 for controls (CTL); n = 8 for compensated cirrhotic rats; n = 7 for decompensated cirrhotic rats. **(b)** Spearman correlation between microbial load and relative abundance of Bacteroidetes (rho = -0.637, p = 0.004) and between microbial load and relative abundance of Proteobacteria (rho = 0.673, p = 0.002) in the MLN.

Correlation between microbiome and cytokine levels

The progression towards decompensated cirrhosis is associated with a high production of pro-inflammatory cytokines such as TNF-α, IL-17 and IL-6 as well as anti-inflammatory cytokines such as IL-10[25, 26]. Using ELISA, we measured serum and MLN levels of these cytokines. To evaluate a possible correlation between the inflammatory status and the microbial community composition, we used the Spearman correlation test to associate levels of the pro-inflammatory cytokine IL-17 in MLNs and the ratios of systemic IL-6/IL-10 and systemic TNF-α /IL-10 with the relative abundance of microbial groups in MLNs. We found a positive

correlation between the IL-6/IL-10 ratio and the relative abundance of *Escherichia* (rho = 0.79, p = 9.2 e-5; Spearman test) and a positive correlation between the TNF-α /IL-10 ratio with the relative abundance of Escherichia (rho = 0.57, p = 0.01; Spearman test) and *Candidatus Arthromitus* (rho = 0.72, p = 0.001; Spearman test) (**Figure 9**).

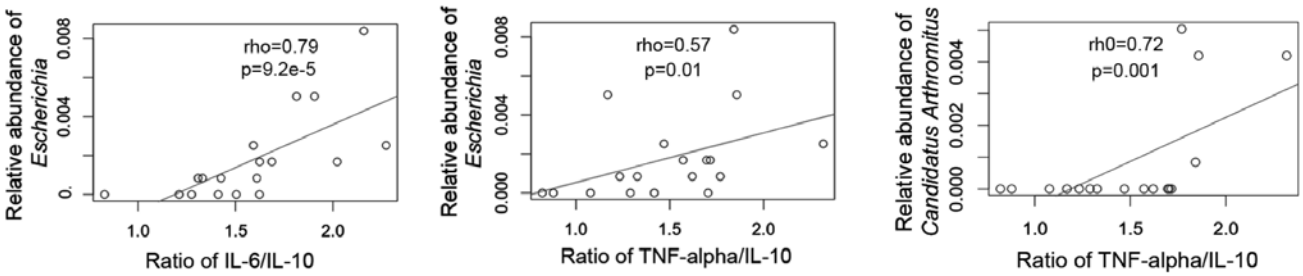

Figure 9. Correlation between pro-inflammatory cytokines and relative abundance of genera in MLNs. Positive Spearman correlations were found between the ratio of systemic IL-6/IL-10 and *Escherichia* and between the ratio of systemic TNF-α/IL-10 and *Escherichia* and *Candidatus Arthromitus*.

We also found a positive correlation between levels of IL-17 and several different microbial genera such as those belonging to Proteobacteria (*Pseudomonas, Burkholderia* and *Sutterella*) and *Candidatus Arthromitus* and negative correlations between levels of IL-17 and *Parabacteroides* and *Coprococcus* (**Figure 10**).

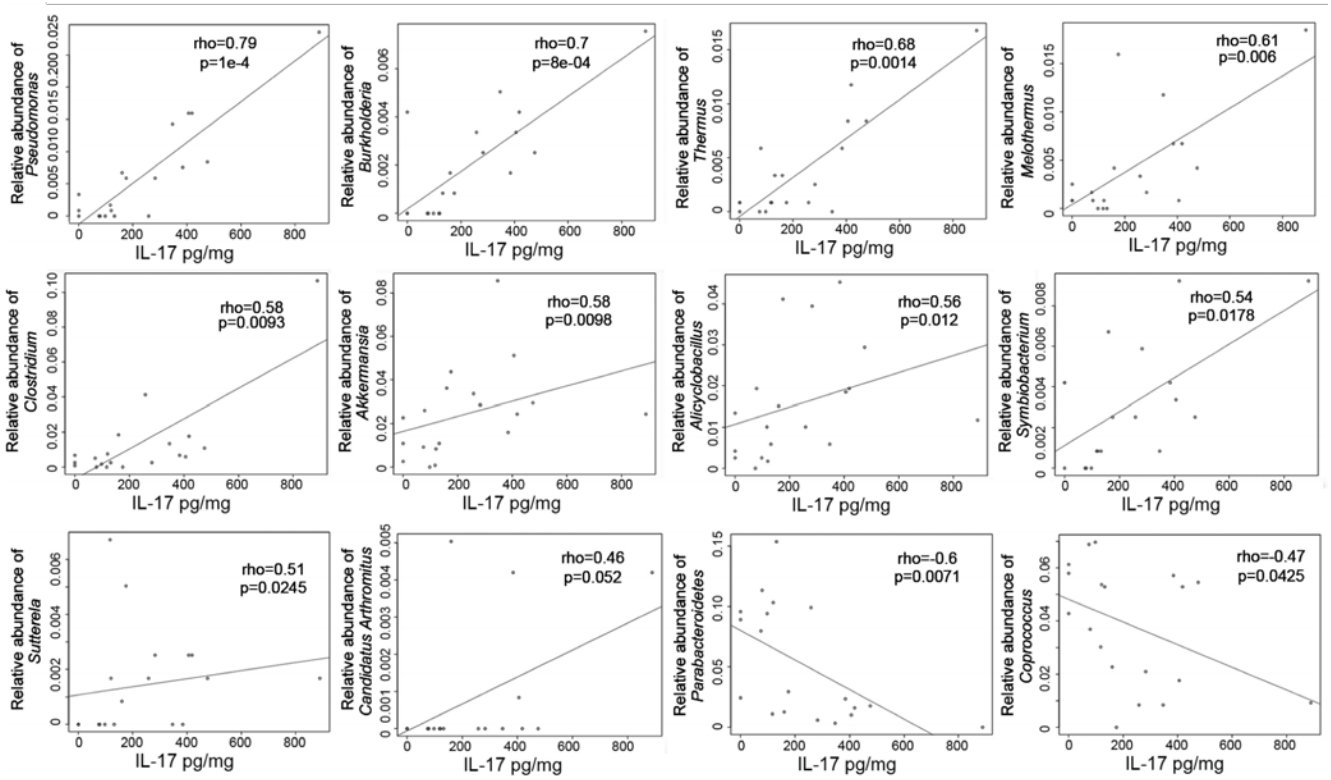

Figure 10. Correlation between pro-inflammatory cytokines and relative abundance of genera in MLNs. Spearman correlations between IL-17 levels measured in MLNs and relative abundance of genera detected in MLNs.

Is *Candidatus Arthromitus* present in human samples?

As for *Candidatus Arthromitus*, *Escherichia* could be detected in ICC, MLN, AF but at the difference with *Candidatus* it could also be detected in blood samples of rats (Fig. 7a). To evaluate whether Escherichia and *Candidatus Arthromitus* could also be involved in the progression of cirrhosis in patients, we tested for the presence of these genera in blood (n = 13) and ascitic fluid (n = 8) from cirrhotic patients and stool from healthy subjects (n = 17) as well as cirrhotic patients (n = 14) that were collected and analysed from a previous study[19]. We also looked for the presence of these genera in the published sequence data from 1016 faecal samples obtained from 977 healthy UK subjects[27]. While *Escherichia* was detected in 84% of the faecal samples (Fig. 7b), *Candidatus Arthromitus* was undetectable in any of the tested samples.

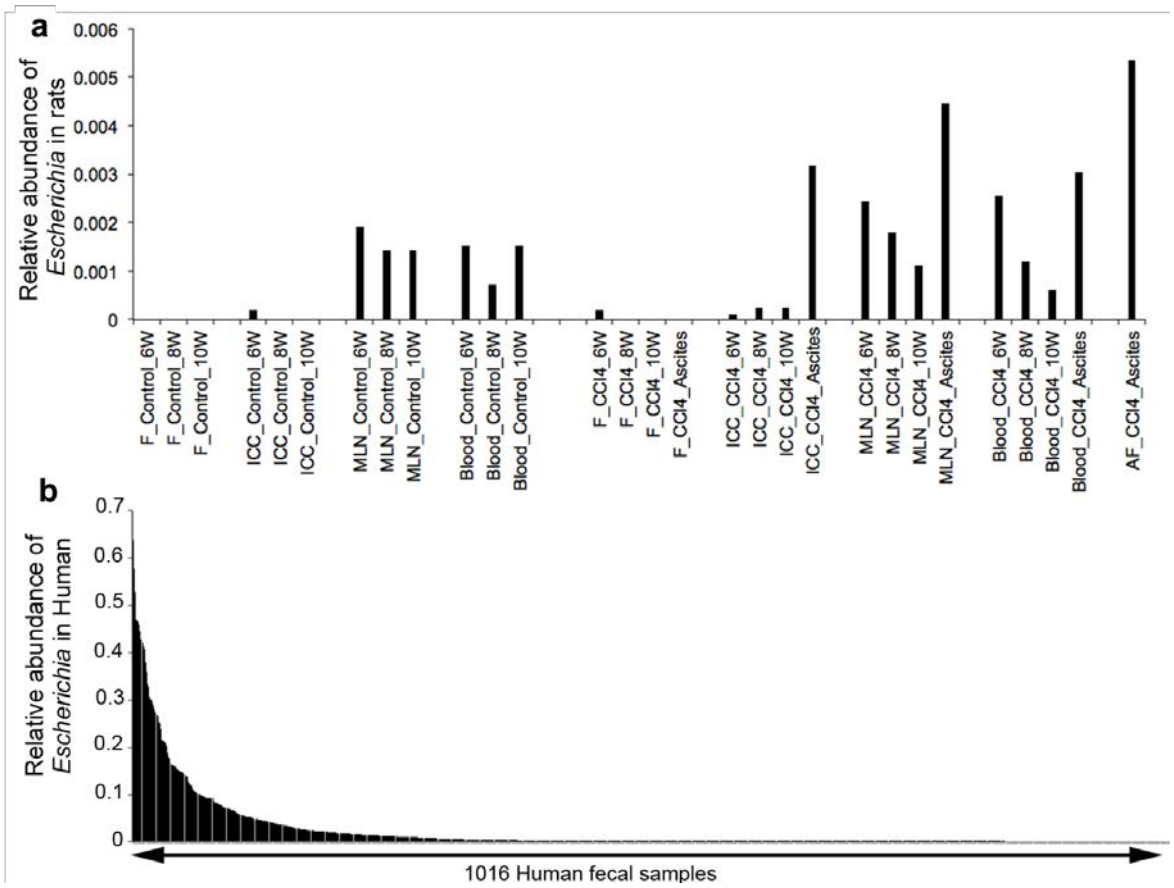

Figure 11. Detection of 16S sequences of Escherichia. (a) In rat samples, **(b)** and human faecal samples from published sequence data from 1016 faecal samples obtained from 977 healthy UK subjects.

DISCUSSION

In this study we show that microbiome changes in intestinal sites are associated with microbial shifts in the MLNs as well as an increase in cytokine production that correlated with disease progression in rats with experimental cirrhosis. The main results of the present work are the characterisation of the sequential changes of the microbiome in the progression of cirrhosis and in particular, the crosstalk between the microbiome and the host immune system using a rat model in a longitudinal and multi-body sites study setting.

Loss of intestinal barrier function, dysbiosis and systemic immune dysfunction characterise cirrhosis.[11] BT is a result of a loss of intestinal barrier function and is considered to be a biomarker for cirrhosis progression and decompensation in which intestinal bacteria travel by paracellular transport through the permeable epithelial cells to the portal vein, the liver and systemic circulation causing an inflammatory response.[7] It has been demonstrated that microorganisms could also be transported by dendritic cells from the intestines to the MLNs via the lymphatic system.[8,28,29] Comparing the composition and function of intestinal and extra-intestinal body sites, our findings suggest a loss of barrier function in which a specific microbial community, particularly Proteobacteria and Actinobacteria, was transported from the intestine to MLNs, blood and AF which caused an induction of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-17 in rats. This outcome was associated with disease progression and decompensation with ascites formation.

It has been suggested that dysbiosis is a major driver of cirrhosis and is also a major contributor to bacterial translocation to MLNs in cirrhosis animal models.[7,30] In the present study, we found more microbiome alterations in ICC than in faecal samples of rats with decompensated cirrhosis. This suggests that faeces analysis may not be sufficient to uncover all of the microbiome alterations that occur in cirrhosis. This is in line with previous studies in patients suggesting that dysbiosis in advanced cirrhosis mainly occurs in upper areas of the intestine such as the ileum,[7] as well as with the finding that microbiota analysis in faecal samples do not accurately represent dysbiosis in sigmoid mucosal samples obtained by biopsy during sigmoidoscopy.[31] In the present study, CCl_4-induced rats showed in their ICC samples an attempt of beneficial groups of bacteria to displace pathobionts. A pathobiont is defined as a symbiont that under certain circumstances becomes a pathogen.[32] This compensation failed when inflammation increased, and rats developed ascites. Therefore, a follow up of

Erysipelotrichia and Betaproteobacteria could be a good indicator of the progression of cirrhosis severity in this experimental model.

Bajaj *et al*.[3] characterised the composition of the faecal microbiome in patients with compensated and decompensated cirrhosis and also evaluated the stability of the microbiome composition at two time points within intervals of 6 months. Our findings confirmed their claim that the level of dysbiosis in stool samples was associated with cirrhosis severity whereas a relatively stable microbiome composition over time was associated with disease stability. Additionally, the use of an animal model allowed us to unravel a possible crosstalk between the microbiome in MLNs and the immune system that was confirmed through a correlation analysis between microbial genera identified in MLNs and levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-17 in MLNs or blood. Moreover, our results are congruent with a comprehensive study that analysed 29 different cytokines from 522 cirrhotic patients indicating that systemic inflammation is likely the underlying cause of decompensation and acute-on-chronic liver failure in cirrhosis.[5]

We also observed a positive correlation between the abundance of Escherichia, belonging to the Proteobacteria phylum, in MLNs and pro-inflammatory cytokines, which confirms the results of previous studies using culture techniques and PCR of the 16S rRNA gene[33,34] making *Escherichia* a potential biomarker for cirrhosis progression.[35] We have further demonstrated that an increase in microbial load was associated with an increase in Proteobacteria in the MLNs of decompensated cirrhotic rats compared to controls and compensated cirrhotic rats. Additionally, our findings from this current study showing an increased microbial function involved in lipid metabolism as well as our previous study that demonstrated increased microbial translocation in advanced cirrhosis,[19] are consistent with the results of Clark *et al*.[36] in which non-pathogenic enteric bacteria, such as *Escherichia coli* strain C25, interact with intestinal epithelial cells in a complex way that regulates immune responses and could become invasive under inflammatory conditions. Such bacteria can cross the intestinal epithelium by exploiting lipid rafts-mediated transcytotic pathways which may precede cytokine-induced disruption of tight-junctions.

Furthermore, we observed that *Candidatus Arthromitus*, a genus from the Firmicutes phylum positively correlated with IL-17 levels, was found in higher abundance in the MLNs of rats with decompensated cirrhosis compared to control rats and compensated cirrhotic rats. This genus was detected in all type of samples except in faeces, which again confirms that the use of stool

samples would not allow the detection of this genus in decompensated state. *Candidatus Arthromitus* is a SFB that can induce the development of multiple adaptive immune responses and in particular Th17 cells in the small intestinal lamina propria of mice.[37,38] Th17 cells, that produce the effector cytokine IL-17, are potent inducers of tissue inflammation and have been associated with the pathogenesis of many immune mediated-diseases.[39] Therefore, *Candidatus Arthromitus* could play an important role in inducing inflammation, which may lead to a decompensated state. This genus is also well known to be refractory to in vitro culture technique,[40,41] which explains why it has not been uncovered in previous studies using traditional culture techniques. However, from our analysis, *Candidatus Arthromitus* was undetectable in many tested human samples, which suggests that another microbial group could be involved in the release of inflammatory cytokines in humans.

Since this work was performed on animals, we may not be able to extrapolate all the findings to cirrhotic patients, particularly in terms of the involvement of specific genera such as *Candidatus Arthromitus*, as the microbiome could present differences in its composition and function. However, our study should pave the way for the search for an equivalent genus to *Candidatus Arthromitus* in humans that is involved in inducing inflammation in cirrhotic patients.

We have demonstrated that microbiome changes in intestinal sites reflect changes in the MLNs microbiome, associating inflammation with an advanced stage of decompensated cirrhosis.

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GENERAL DISCUSSION

GENERAL DISCUSSION

To summarise, in the present thesis, in an animal model of CCl_4-induced cirrhosis in rats, we showed that upon CCl_4-induction, pathobionts attempted to displace symbionts in ICC samples, and finally symbionts failed to compensate pathobionts when rats developed ascites. These changes in intestinal sites were associated with microbial shifts in the MLNs. An increased microbial load was positively correlated with the relative abundance of Proteobacteria in the MLN of decompensated rats. Besides, among several genera, *Escherichia* and *Candidatus Arthromitus*, were correlated with elevated levels of pro-inflammatory cytokines. Overall, our findings suggest the important role of the gut-liver-immunity axis in the progression of cirrhosis.

Moreover, we observed the presence of polymicrobial DNA in the serum and ascitic fluid of patients with cirrhosis but scarcely detectable in the serum of healthy controls. The serum microbiome of patients with ascites presented higher levels of LBP, a marker of bacterial translocation, associated with higher diversity and relative abundance of Clostridiales and an unknown genus belonging to the Cyanobacteria phylum compared to patients without ascites. The composition of the faecal microbiome was also more altered in patients with than without ascites.

Cirrhosis is a progressive and chronic condition triggered by several factors and is considered an important cause of morbidity and mortality specially in developed countries.[136] Once the decompensated stage is reached, certain clinical complications such as ascites, infections (including SBP), gastrointestinal bleeding, or encephalopathy appear.[170] There is growing evidence pointing out that gut microbiota aids in the pathogenesis of liver cirrhosis and its complications. Bacterial translocation plays a fundamental role in this pathophysiology contributing at the end to the overall systemic inflammatory milieu.[188,208]

CCl_4 is the most commonly used hepatotoxin to induce liver cirrhosis in the experimental animal, and the repeated administration of CCl_4 is one of the most used experimental models to study the pathogenesis of liver fibrosis, cirrhosis and portal hypertension.[189,209–211] The model of hepatic cirrhosis induced by CCl_4 has considerable advantages, since it is an inexpensive and easily realizable model, has minimal extrahepatic toxic effects and has been used successfully in several species and strains. In comparison with the other experimental models of cirrhosis, the administration of CCl_4 also has the important characteristic that the

hepatic architectural and hemodynamic alterations that it produces are similar to those observed in liver cirrhosis in humans.[210] For these reasons, we decided to use this experimental model in the present thesis, in addition to the study in humans.

Most of the studies performed until now in animal models of cirrhosis have mainly focused on variations in stool and intestinal content microbiome. At the end of the 1990's, a high prevalence of *E. coli* and *Proteus mirabilis*, both belonging to Enterobacteriaceae, was observed in the caecum of rats with CCl_4-induced cirrhosis.[120] However, no significant differences were observed in the intestinal content of CCl_4-treated mice at different chronological points of cirrhosis induction when compared to controls.[212] Even so, it has been stated that the microbiome stability could be a potential biomarker for disease progression, since a marked dysbiosis was found in patients with decompensated cirrhosis while patients with a non-evolving disease course had a more stable microbiota.[213] In our animal model, an increase at the class level of Erysipelotrichia and Betaproteobacteria was observed, as well as an increase of *Sutterella* and *Coprococcus* at the genus level in decompensated rats; these groups of bacteria being possible indicators of disease progression.

Throughout these years, the gut microbiome has been widely studied through faecal samples, but to what extent do the faeces represent the composition of different intestinal sites is not well understood. It has been described that mucosal microbiota is notably different and shows a higher stability over time than that of the lumen.[214] In our study with rats, we have observed more differences between the different stages of cirrhosis in the ICC microbiome than in the faeces. Similar results, in which both types of samples do not cluster, have been previously seen in both: animal models[215] and humans.[216] Moreover, Zoetendal *et al*. described differences between the microbiome of the intestinal mucosa and faecal microbiome in a human non-cirrhotic cohort.[217] In line with these results, when analysing the bacterial composition of the complete gastrointestinal tract of the rat, it was observed that it changed from more aerobic bacteria in the upper parts, to obligate anaerobes in lower parts demonstrating that faecal samples are not always a good representation of the whole gastrointestinal tract microbiome.[218] Differences between sigmoid mucosal and stool microbiome in cirrhotic patients were confirmed by Bajaj *et al*.[219] Chen *et al*. also found remarkable differences in the duodenal mucosal microbiota of cirrhotic patients compared to healthy subjects, confirming that microbial alterations are not limited to the lower digestive tract.[220]

Up to now, very little is known about the microbiome of extra-intestinal sites. Therefore, keeping in mind that a greater dysbiosis is associated with a worsening of the disease, we wanted to take advantage of the use of a rat model to study the microbial composition in MLNs and blood at different stages of the disease. Even though there were only significant differences in the stability of the microbiome in blood, being a lower recoverable amount in rats with ascites compared to those without ascites, dissimilarities in relative abundances of various bacterial groups were found in both MLN and blood at different stages.

Although a greater proportion of bacteria in MLNs of rats with ascites compared to control rats was previously reported through conventional cultures,[221] it was not until the appearance of new molecular technologies that we were able to describe the microbial composition of MLNs in detail. In our preliminary observations, Clostridiales were found to be 6.6-fold more abundant in MLNs in controls than in cirrhotic rats.[155] However, in our present study we found that the relative abundance of *Candidatus Arthromitus*, belonging to Costridiales, was higher in MLNs of decompensated cirrhotic rats. These discrepancies could be mainly due to the different extraction methods performed in both studies and the sequencing technology used, a 454 Life Sciences (Roche) FLX system in the preliminary study versus the Illumina MiSeq technology in the following ones. Other factors to consider could be the differences in the sample size or the updating of the databases used during the bioinformatics analysis. Nevertheless, it should be noted that in our present study it has been observed that an increase in microbial load was associated with an increase in the relative abundance of Proteobacteria in the MLNs of decompensated cirrhotic rats.

As well as dysbiosis, microbial functional changes are thought to play a role in the progression of liver cirrhosis.[222] It is known that non-alcoholic fatty liver disease (NAFLD) is one of the main common causes leading to chronic liver disease and it occurs when there is an excess of lipid deposition in the liver (steatosis).[223] In our study, functions involved in lipid metabolism were found to be increased in the MLNs of decompensated rats. Moreover, the relative abundance of functions targeting the immune system were also increased in this group of rats confirming the involvement of immune responses in the progression of cirrhosis.

Considering BT as a major player in the progression and decompensation of cirrhosis and taking into account that dysbiosis could be an important contributor to this phenomenon, an in-depth study of changes in the intestinal and extra-intestinal microbiota in an animal model could help to elucidate the physiopathological mechanisms of the disease.[177,184,212]

The progression towards an advanced stage of cirrhosis is associated with a high production of pro-inflammatory cytokines like TNF-α, IL-17 and IL-6 as well as anti-inflammatory cytokines such as IL-10.[224] Confirming the pro-inflammatory scenario, Gomez-Hurtado *et al*. showed that the levels of TNF-α and IL-6 were significantly increased in serum of cirrhotic mice and correlated with the decrease of *Clostridium* in intestinal content.[212] Although there is an activation in the production of cytokines with opposed functions, our results confirmed a previous study in which a clear unbalance in favour of pro-inflammatory cytokines was shown, as indicated by the significantly higher IL-6/IL-10 and IL-8/IL-10 ratios observed in cirrhotic patients compared to healthy subjects.[225] Moreover, and in an attempt to study the interplay between microbiota and the immune system we observed a positive correlation between IL-6/IL-10 ratio and the relative abundance of *Escherichia*; and between TNF-α /IL-10 ratio and the relative abundance of *Escherichia* and *Candidatus Arthromitus*, suggesting a relationship between the translocation of specific bacteria to extra-intestinal sites and the induction of an inflammatory response. We also found positive correlation between levels of IL-17 in MLNs and several genera belonging to Proteobacteria and *Candidatus Arthromitus* and negative correlations between levels of IL-17 and *Parabacteroides* and *Coprococcus*.

Indeed, the use of an animal model allows us to obtain samples that are difficult to obtain in humans or require a very invasive method, such as MLNs. However, we must bear in mind that one of the main concerns when working with animal models is if the results obtained are an accurate reflection of what is happening in humans. The choice of an appropriate and reproducible model becomes a crucial part of any experimental design. For instance, we found that abundance in MLNs of *Candidatus Arthromitus*, which was positively correlated with IL-17 levels, was higher in rats with decompensated cirrhosis suggesting its involvement in the induction of an inflammatory response. However, this bacterium was undetectable in a huge set of human faecal samples, ruling out the idea that it could also be involved in the progression of cirrhosis in patients. Even so, the existence of another equivalent microbial group which could be involved in a similar scenario in humans is not illogical.

Thus, since findings in animal models cannot always be extrapolated to humans there exists the necessity to validate the results obtained in previous studies with animals with studies performed in humans, as we have done in the present thesis.

Most of the studies carried out until now in patients with cirrhosis, as with rats, had mainly focused on variations in the stool microbiome. Our study in humans showed, in agreement

with other studies,[213,222] a decrease in faecal microbial diversity as well as a depletion of several autochthonous taxa (unknown Ruminococcaceae, Clostridiales and Peptostroptococcaceae, *Roseburia faecis* and *Alistipes putredinis*) in patients with cirrhosis compared to controls. Nevertheless, we did not find significant increase in potential pathogenic bacteria such as Enterobacteria, but only a trend towards an increase in Proteobacteria or Streptococcaceae. This finding could be related to the relatively preserved liver function of the patients included in the study, as shown by the low values of the Child-Pugh and MELD scores even in patients with ascites. Moreover, our results in which the stool microbiome of patients with ascites at the species level presented a depletion of several commensal bacteria compared to those without ascites are also in line with previous findings describing that a higher level of dysbiosis was associated with a worsening of the disease.[213]

Similar results were previously highlighted by Chen *et al*., showing a decrease in commensal taxa such as Lachnospiraceae as well as an increase in potential pathogenic bacteria like Enterobacteriaceae belonging to Proteobacteria phylum among others, were the main characteristics observed in the faecal microbiota of cirrhotic patients. It is thought that these changes could contribute to the development of complications in cirrhosis.[188]

One of the known beneficial effects of bacteria belonging to the Lachnospiraceae family is its association with the production of SCFAs, especially butyrate.[226,227] These molecules are the result of microbial carbohydrate fermentation; acetate, propionate and butyrate being the most abundant.[10] Among the different SCFAs, butyrate is required for the integrity of the epithelial barrier through the regulation of the TJs.[228,229] Moreover, new evidences have raised about the role of SCFA in the regulation of the immune system and inflammatory response.[230,231] An increased inflammatory response has been associated with a reduction of the *Clostridium* groups and therefore favouring bacterial translocation and liver injury.[212] Species belonging to the *Clostridium* group have been also considered as butyrate-producing bacteria,[232] therefore a suppression of specific SCFA production might damage the intestinal barrier and promote a pro-inflammatory response.

Currently, one of the methods used to define changes in the intestinal microbiota of cirrhotic patients is the Cirrhosis Dysbiosis Ratio (CDR), a ratio that allows the evaluation of the relative good and bad taxa abundance and was introduced by Bajaj *et al*.[213] In agreement with their observations, Sarangi *et al*. found that CDR was significantly lower in cirrhotic patients compared to controls.[233] These changes in stool microbial composition have been associated

with disease progression and endotoxemia[188,234] as well as with changes in mucosal microbiome which might be in turn related with encephalopathy.[219,220]

Very little is known about the microbiome of extra-intestinal sites in patients with cirrhosis or the underlying mechanisms that involve gut microbiota in the prognosis. So, in an attempt to shed light on this issue, using high throughput sequencing techniques we tried to define serum and ascitic fluid microbiome. For the first time, we showed the presence of polymicrobial DNA in both type of samples of cirrhotic patients. Even though standard microbiological methods revealed negative bacterial growth for serum and ascitic fluid, we were able to detect sequence data of approximately 89% and 73% of the samples, respectively.

Our results showed that serum and ascitic fluid microbiome shared more than 80% of similarity with faecal microbiome at the genus level. At the phylum level the three sample types were dominated by Firmicutes and Bacteroidetes. One of the first studies that reported the presence of bacterial DNA in these "low biomass" samples was performed by Such *et al*. in hospitalised patients in which mostly *E. coli* was detected in serum and ascitic fluid in a low percentage of patients (around 32%).[235] With the presence of bacterial DNA in extra-intestinal sites considered as molecular evidence of bacterial translocation[186], several subsequent studies were encouraged to try bacterial detection in these type of samples. Thus, other authors detected bacterial DNA among 20-30% of the patients with cirrhosis, and DNA was mostly monomicrobial with *E. coli, Klebsiella* or *Staphylococcus aureus* as the most frequently identified microorganisms.[236–239] In contrast, Sersté *et al*. achieved the detection of bacterial DNA in a still lower number of subjects.[240] These divergences could be due to the different characteristics of the recruited subjects, and more probably to differences in the methodologies used (16S rDNA PCR-based method or multiplex PCR).

With the application of the Next Generation Sequencing (NGS) techniques, studies characterising a polymicrobial microbiome in blood and ascitic fluid began to appear. For instance, Feng and co-workers using a 16S PCR approach and subsequent Illumina sequencing were able to detect more than one genus being *E. coli* the most prevalent, but only in one sample.[241] Moreover, a recent study using a specific microbial DNA qPCR Array described the blood microbiome in decompensated cirrhotic patients in agreement with the results of our study with a few differences in the genera found.[242] Regarding dysbiosis in ascitic fluid, Rogers *et al*. succeeded in identifying it with an increase in the abundance of Enterobacteriaceae.[243]

It is known that BT could play an important role in the pathophysiology of liver cirrhosis and its complications. For the first time, we were able to demonstrate the presence of polymicrobial DNA in extra-intestinal sites in both humans and rats through new sequencing technologies. Besides, in agreement with other studies[207], we demonstrated that the level of BT is positively correlated with an advanced status of the disease by a higher concentration of LBP in patients with ascites.

The relationship between BT and the progression of cirrhosis could be explained by the activation of the immune response, which can lead to an epithelial barrier dysfunction and therefore to a perpetuation of BT. Moreover, our results showing association between the translocation of specific bacteria and the induction of the immune system are in line with the hypothesis of Wiest *et al*.[177]

Our work allowed us to obtain results that answer some of the initial objectives of the present thesis. Even so, we must take into account the limitations that have arisen throughout the studies and the improvements that should be done for future studies.

One of the major limitations of our study is the fact that we did not distinguish the presence of viable from non-viable bacteria. Cangelosi *et al*. highlighted the importance of differentiating between dead and live bacteria to avoid bias during the sequencing analysis due to the persistence of "cadaver cells" for a while after some treatments.[244] Thus, it would be interesting to use methods that determine bacterial viability, such as the use of propidium monoazide (PMA) which penetrates inactivated cells and inhibits PCR amplification, microscopy-based live/dead staining or molecular viability testing (MVT) which correlates viability with the ability to rapidly synthesise a macromolecule (a species-specific rRNA precursor, or pre-rRNA) in response to a brief nutritional stimulus.[245] Moreover, Bajaj *et al*. used bacterial RNA since active RNA synthesis could imply active bacteria.[246]

Moreover, it has been observed that the analysis of the faecal microbiota alone might be unrepresentative of the precise gut mucosal microbiota and its relationship with the host.[218,219] Thus, further studies including intestinal mucosa biopsies would be necessary to better understand the interplay between gut microbiota and the host in liver cirrhosis.

When working with low biomass samples, the possibility of contamination generated during the extraction and PCR amplification has to be taken into account. Therefore, strict protocols to maintain sterile conditions throughout the process were applied for sample collection, DNA

extraction, and PCR amplification. For DNA extraction, chemicals such as DNA terminator (Biotools, B & M Labs, Spain) were used to degrade any trace of contaminant DNA in laboratory equipment. Furthermore, the addition of negative controls (blanks) throughout the process as well as the reduction in the amount of *Taq* polymerase used[247] is considered essential in order to be able to subtract the sequences of the potential contaminants. This is consistent with our findings in which *Escherichia* belonging to the Proteobacteria phylum was found in the extraction and PCR blanks.

In order to describe the real involvement of the human microbiome in health and disease, it is very important to have standardised methods to obtain comparable data within worldwide studies. One of the limitations described throughout the discussion is the use of different methodologies as a possible factor of the inconsistencies found in some results of various studies. The International Human Microbiome Standards (IHMS) works towards the achievement of these goals [http://www.microbiome-standards.org/].[248]

Both studies discuss the possibility of using specific alterations in the microbiome of different types of samples as indicators of the progression of the disease. This is in line with the statements of the European Association for the Study of the Liver (EASL) Special Conference 2013 about the potential of new biomarkers to improve preventive and therapeutic strategies in cirrhosis.[173]

As previously described, the mechanisms involved in BT are a compromised immune system, alterations in gut microbiota and impaired intestinal barrier. Therefore, all these three mechanisms are possible targets to prevent BT, and any measure that improves one or several of them could be used as a potential treatment to prevent cirrhosis progression and the development of complications. Regarding the alterations in gut microbiota, bacterial overgrowth is the most commonly associated to BT[120,178] and has been attributed to a delay in the intestinal motility and therefore, a prolonged intestinal transit time.[249] Thus, administration of prokinetic agents such as mosapride has been studied as a preventive therapy for BT in CCl_4-induced cirrhotic rats.[250] Besides, due to the growing emergence of bacterial resistance to antibiotics, the mechanism by which a reestablishment of intestinal microbiota homeostasis can be favoured had to be modified. In addition to beta-blockers, bile acids or antioxidants[251], several studies have observed a beneficial effect of probiotics on the progression of liver cirrhosis by their impact on BT.[251–253]

CONCLUSIONS

CONCLUSION

The results of the present PhD work, despite the limitations, offer valuable insights to the involvement of bacterial translocation in the progression of liver cirrhosis, leading to the following conclusions:

1. Microbiome changes in intestinal sites are associated with microbial shifts in the mesenteric lymph nodes in a rat model of cirrhosis.

2. Specific genera in serum and mesenteric lymph nodes were correlated with elevated levels of pro-inflammatory cytokines associated with an advanced stage of decompensated experimental cirrhosis.

3. The correlation of a specific bacterial group with inflammatory response in rats and undetectable in human faecal samples, suggest that another microbial group could be involved in the release of inflammatory cytokines in humans.

4. The presence of bacteria in extra-intestinal sites and the higher levels of serum lipopolysaccharide binding protein in patients with cirrhosis support the role of bacterial translocation in the disease progression.

5. Serum and ascitic fluid of patients with cirrhosis contain a complex and specific microbial community but barely detectable in the serum of healthy controls.

6. Alteration of the serum and faecal microbiome composition could be considered indicators of cirrhosis progression.

Further ameliorated studies will be needed to validate our findings and go in depth in the bacterial translocation phenomenon to improve prognostic and therapeutic strategies.

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ANNEXES

ANNEX 1. Publication related to the thesis. Cuenca *et al.* 2013 "Microbiome composition by pyrosequencing in mesenteric lymph nodes of rats with CCl4-induced cirrhosis."

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Microbiome Composition by Pyrosequencing in Mesenteric Lymph Nodes of Rats with CCl_4-Induced Cirrhosis

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Key Words
Carbon tetrachloride · Cirrhosis · Microbiota · Mesenteric lymph node

Abstract
Background: The cross talk between the gut microbiota and the immune system, which is essential to maintain homeostasis, takes place at the intestinal lymphoid tissue such as the mesenteric lymph nodes (MLNs). Here, we investigated the presence of bacterial DNA in MLNs of control and cirrhotic rats and its relationship with inflammatory responses. ***Methods:*** The MLN microbiome of cirrhotic rats with ascites, which was induced by carbon tetrachloride (CCl_4), was compared to that of control rats using quantitative real-time PCR and pyrosequencing of the 16S rRNA gene. Cytokines in blood samples were assessed by ELISA. ***Results:*** Unexpectedly, sequence analysis revealed a high microbial diversity in the MLNs of both control and cirrhotic rats with Proteobacteria as one of the most dominant phylum. CCl_4-induced liver injury was not associated with a change in bacterial load, but it was linked to a decrease in microbial diversity ($p < 0.05$) and alterations in the microbial community in MLNs. A high proportion of *Bifidobacterium animalis* was also positively correlated with elevated interleukin-10 expression ($p = 0.002$, false discovery rate = 0.03, $r = 0.94$). ***Conclusions:*** For the first time, the high microbial diversity observed in MLNs of both controls and CCl_4-induced cirrhotic rats provides evidence that bacterial translocation is more than a mere dichotomic phenomenon.

Silvia Cuenca and Elisabet Sanchez are co-first authors. The genetic sequence data will be submitted to the GenBank database upon publication.

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Introduction

Cirrhosis is the final phase of chronic liver disease, in which inflammation is associated with dying hepatic cells and fibrosis, which lead to poor liver function and portal hypertension.

Bacterial translocation is defined as the migration of viable bacteria or their products from the gastrointestinal tract to mesenteric lymph nodes (MLNs) and other extraintestinal sites, such as the systemic circulation or extraintestinal organs [1]. MLNs are a vital part of the immune system. They carry specialized cells that trigger immune system responses and play a crucial role in the interplay with intestinal microbiota. Bacterial translocation has been postulated as a main mechanism in the pathogenesis of spontaneous infections in cirrhosis and also in the hyperdynamic circulatory state, a key factor in the pathogenesis of portal hypertension, ascites development and other cirrhosis complications [2–7].

Until recently, bacterial translocation in cirrhosis was considered a dichotomic and mostly monomicrobial phenomenon [8, 9]. However, recent studies using techniques of advanced molecular biology have questioned this interpretation [10, 11]. Bacterial DNA and also live bacteria have been detected in the MLNs of healthy mice, where they have been transported from the digestive tract via dendritic cells [12–14]. Using a 16S rRNA gene clone library analysis by conventional Sanger technique to analyze the bacterial composition, MLNs were found to be dominated mainly by *Pseudomonas* and *Alcaligenes* spp., which belong to the Proteobacteria phylum [14]. These results confirmed that bacterial translocation into the MLNs can be polymicrobial.

Dysbiosis (microbial community alteration) in fecal microbial communities has been evidenced in patients with liver cirrhosis, where Bacteroidetes phylum was significantly reduced while Proteobacteria and Fusobacteria phyla were highly enriched [15]. Members of the Proteobacteria and Bacteroidetes phyla have also been associated with inflammation in cirrhotic patients presenting a hepatic encephalopathy complication [16]. However, to our knowledge, no study using pyrosequencing has shown dysbiosis in MLNs in liver disorders, where the interplay between microbiota and the immune system could play a crucial role in the inflammation and therefore in the evolution of the disease.

Chronic liver damage induced by carbon tetrachloride (CCl_4) administration in rodents is an experimental model that has been widely used to study the pathogenesis of cirrhosis, ascites and bacterial translocation [17].

In the present pilot study, using a rat model of cirrhosis and a high-throughput sequencing technology, we evaluated the microbial composition of MLNs of cirrhotic and control rats, and its relationship with immune responses in the systemic circulation. Our findings disclose the dysbiosis down to the species level and link an anti-inflammatory cytokine to a specific bacterial taxon.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 35–49 g were purchased from Harlan Laboratories (Indianapolis, Ind., USA) and provided by Research Models and Services Production (Udine, Italy). Rats after weaning and their mothers were fed a rodent chow diet (2018S; Teklad, Madison, Wisc., USA). After a 1-week quarantine, all animals were placed in individual cages and kept at a constant room temperature of 21°C, exposed to a 12-hour light:12-hour dark cycle and allowed free access to water and rodent chow (A04; SAFE, Augy, France). There was no contact between rats neither through water, chow or feces of other animals. The study was approved by the Animal Research Committee at the Institut de Recerca of Hospital de la Santa Creu i Sant Pau (Barcelona) and by the Department of Agriculture, Livestock and Fisheries of the Generalitat de Catalunya (Departament d'Agricultura, Ramaderia i Pesca). Animal care complied with the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*.

Induction of Cirrhosis and Study Groups

Cirrhosis was induced as previously described [18]. When rats reached a weight of 200 g, they were administered weekly doses of CCl_4 (Sigma-Aldrich, St. Louis, Mo., USA) intragastrically using a sterile pyrogen-free syringe (ICO plus 3 Novico Médica, S.A., Barcelona, Spain] with an attached stainless-steel animal feeding tube (Popper and Sons, New Hyde Park, N.Y., USA) without anesthesia. The first dose of CCl_4 was 20 µl, and subsequent doses were adjusted on the basis of changes in weight 48 h after the previous dose, as reported previously [19]. When cirrhotic rats presented ascites, the dose of CCl_4 was maintained at 40 µl. Non cirrhotic rats receiving tap water were used as controls.

Laparotomy

When ascites was suspected in cirrhotic rats based on the increase in abdominal girth, a paracentesis was carried to confirm the presence of ascitic fluid. Paracenteses were performed under air anesthesia with isofluorane (Forane®; Abbott, Madrid, Spain) in sterile conditions, and approximately 0.1 ml of ascitic fluid were removed. One week later, a laparotomy was carried out on all cirrhotic rats, and in control rats, laparotomy was performed at the corresponding weeks.

For laparotomy, rats were anesthesized with 10 mg/kg xylazine (Rompun®; Bayer, Kiel, Germany) and 50 mg/kg ketamine (Ketolar®; Parke-Dawis, Madrid, Spain) in sterile conditions. In brief, the abdominal fur was removed with a depilatory cream and the skin was sterilized with iodine. The abdomen was then opened via a 4-cm median incision, and the remaining fluid was removed. Samples of ascitic fluid (and pleural fluid if present) were collected

for bacterial culture. Ten MLNs were aseptically and randomly collected from the ileocecal area, weighed and homogenized in sterile saline solution for later analyses. Blood was collected from the vena cava into a sterile EDTA-containing BD Vacutainer® tube (BD Biosciences, San Jose, Calif., USA) without additives, centrifuged and stored at –80°C until later analysis. Liver and spleen tissue was sampled for histological evaluation and bacterial culture and also frozen at –80°C. Rats were then euthanized with intravenous sodium thiopentate (Pentothal®; Abbott Laboratories).

Bacterial Culture

We performed bacterial cultures of samples of MLN homogenate, spleen and liver of cirrhotic and control rats, and ascites and pleural samples were obtained from cirrhotic rats. All the samples were inoculated on Columbia blood agar, Columbia CNA agar and chromogenic medium (CPS ID2; BioMérieux, Marcy-l'Etoile, France). Isolates were identified on the basis of their growth and morphology. Gram stain, catalase, coagulase or oxidase assays were performed when required.

Biochemical Analysis

Serum supernatants were tested for interleukin (IL)-6 and IL-10 using an ELISA kit (PeproTech, London, UK) according to the manufacturer's instructions. Cytokines were quantified with standard curves provided by the kit. The detection limits were set at 30 pg/ml for both cytokines.

Genomic DNA Extraction

From each rat, a sample of the MLN homogenate was subjected to genomic DNA extraction using the DNeasy® blood and tissue kit (Qiagen, Madrid, Spain) according to the manufacturer's recommended protocol.

Assessment of Microbial Load

To assess microbial load, we used extracted DNA to amplify the V4 region of the 16S rRNA gene by quantitative real-time PCR (qPCR) using the following primers: V4F_517_17 (5′-GCCAGCAGCCGCGGTAA-3′) and V4R_805_19 (5′-GACTACCAGGGTATCTAAT-3′). qPCR was performed with the 7500 fast real-time PCR system (Applied Biosystems, Foster City, Calif., USA) using optical-grade 96-well plates. PCR was carried out in a total volume of 25 µl using the power SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) containing 6.25 pmol/µl of each of the above-cited universal forward and reverse primers. The reaction conditions for DNA amplification were as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate and mean values were calculated. Data were analyzed using Sequence Detection Software (version 1.4) supplied by Applied Biosystems. The target DNA copy number was determined by comparison with serially diluting standards (10^1 to 10^6 copies of plasmid DNA containing the amplicon) running on the same plate, as previously described [20]. Bacterial load was expressed as copy numbers of 16S rRNA gene per gram of MLN.

Analysis of Microbial Community Composition

To analyze the microbial community composition of the MLNs, we performed a 16S rRNA gene survey. For this purpose, extracted genomic DNA was subjected to PCR amplification of the V4 region of the bacterial and archaeal 16S rRNA gene. The V4 primer pairs used in this study are expected to amplify >95% of the archaeal and bacterial domains according to our analysis done using PrimerProspector software [21]. The 5′ ends of the forward (V4F_517_17) and reverse (V4R_805_19) primers targeting the 16S gene were tagged with the following specific sequences for pyrosequencing: 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-(multiplex identifier)-(GCCAGCAGCCGCGGTAA)-3′ and 5′ CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-(GACTACCAGGGTATCTAAT)-3′. Tag pyrosequencing was performed using multiplex identifiers of 10 bases, which were provided by Roche (Nutley, N.J., USA) and specified upstream of the forward primer sequence. Standard PCR [1 unit of Taq polymerase (Roche) and 20 pmol/µl of the forward and reverse primers] was run in a Mastercycler gradient (Eppendorf, Hamburg, Germany) at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 56°C for 20 s, 72°C for 40 s and a final cycle of 72°C for 7 min. As a negative control for the PCR, H_2O was used instead of the template. The integrity and specificity of the 16S rRNA V4 amplicons were confirmed by microcapillary electrophoresis using an Agilent 2100 Bioanalyzer with the DNA 1000 kit. Subsequently, the amplicons were sequenced on a 454 Life Sciences (Roche) FLX system (Scientific and Technical Support Unit, Vall d'Hebron Research Institute, Barcelona, Spain) following standard 454 platform protocols. To rule out possible contamination, we also confirmed that no amplicon was produced after PCR of spleen samples of all rats for which we were not expecting bacterial DNA amplification.

The sequences were analyzed using the QIIME (Quantitative Insights into Microbial Ecology) pipeline [22]. From the pyrosequencing experiment, 25,579 high-quality sequences with an average of 290 bp were recovered from all the samples (with an average of 3,654 sequences per sample) after filtering high-quality readings, as previously described [23]. From all samples, using a 97% similarity cutoff, we obtained 412 taxa (or molecular species). After removing taxa with low abundance (i.e. we considered only taxa that were represented by at least 0.2% of the sequences in a sample), we recovered 143 microbial taxa.

Rarefaction analysis was done for all samples, with 10 repetitions using a step size of 100 (100–2,700 sequences per sample). For β diversity analyses, which examine changes between microbial communities, sequence data were normalized at 2,718 sequences per sample. This number was chosen to avoid the exclusion of samples with a lower number of sequence readings from further analysis. The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis was performed on pairwise unweighted UniFrac distances [24].

Statistical Analysis

Differences in microbial load between groups were analyzed using the nonparametric Mann-Whitney U test, with values of $p < 0.05$ considered statistically significant for all tests. We used the otu_category_significance.py script from the QIIME pipeline to test the taxa associated with experimental cirrhosis status and cytokine concentrations. This analysis provided the false discovery rate (FDR) value, which is defined to be the false discovery rate of the p value (corrected p value) and is considered significant when <0.1 [18]. In order to determine the microbial taxon or group of taxa that is increased or decreased in the cirrhotic rats versus controls, we performed analysis of variance (ANOVA) to compare differences between group means.

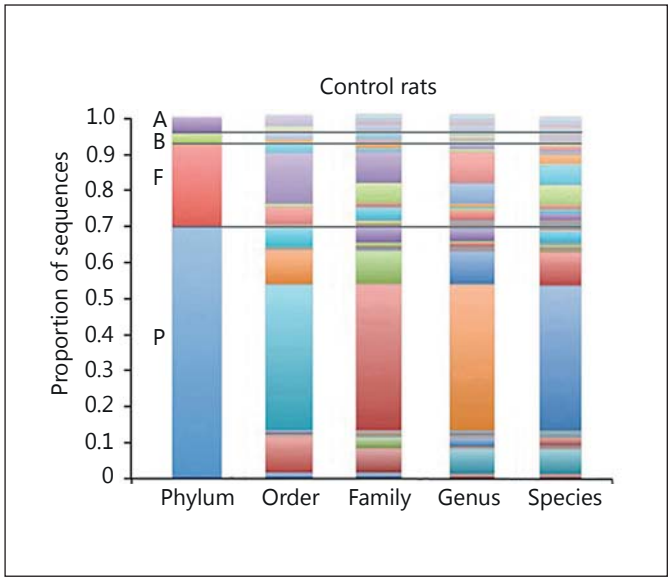

Fig. 1. High microbial diversity in MLNs. The microbial diversity of MLNs of control rats (average proportion of sequences of the 4 healthy rats) is shown at several taxonomic levels based on the analysis of 16S RNA gene sequences. P = Proteobacteria; F = Firmicutes; B = Bacteroidetes; A = Actinobacteria.

Results

We included a total of 7 rats: 3 with CCl_4-induced cirrhosis and 4 control rats. In cirrhotic rats, to confirm the presence of ascites paracentesis was performed 14, 17 and 21 weeks after the first CCl_4 dose, respectively.

Bacterial Culture

Cultures of the spleen, liver and MLN homogenate were negative in the 4 control rats. Cultures of liver, spleen, ascites and pleural fluid tissue from the 3 cirrhotic rats were also negative. Cultures of homogenates from MLNs were positive only in 1 (rat G24) of the 3 cirrhotic rats. In this rat, *Escherichia coli* was isolated.

High Microbial Diversity in the MLNs of All Control Rats

From the sequence data analysis, we unexpectedly detected an interestingly high microbial diversity in all MLNs of control rats. The microbial community was dominated by 4 bacterial phyla: Proteobacteria (61.5%, SD = 9.7%), Firmicutes (28.2%, SD = 10%), Actinobacteria (5.5%, SD = 0.8%) and Bacteroidetes (4.4%, SD = 1.3%). At a lower taxonomic level, we identified an average of 10 microbial classes, 28 orders, 63 families, 82 genera and 88 bacterial taxa. No Archaea were found in our control rats (fig. 1). Among the Proteobacteria phylum, an unknown species from the Enterobacteriaceae family was the most abundant taxon (36%, SD = 8%).

Dysbiotic Microbiome in Cirrhotic Rats

Microbial DNA was detected in MLN homogenate of all cirrhotic rats. The community diversity was lower in cirrhotic than in control rats, which was estimated by 3 indexes: Chao1, phylogenetic diversity and the number of taxa observed ($p < 0.05$; Mann-Whitney test; fig. 2). Control rats presented individually higher numbers of bacterial taxa: 75 (G31), 74 (G32), 78 (G35) and 76 (G37) than cirrhotic rats: 55 (G24), 51 (G25) and 45 (G28). However, this finding was not associated with a difference in the microbial load between the two groups, as evaluated by qPCR (fig. 3).

We used the UPGMA method based on the unweighted UniFrac metric to compare samples. This analysis measures the phylogenetic distance between bacterial communities in a phylogenetic tree, thereby providing a measure of similarity among microbial communities present in distinct samples [24]. As shown in the figure 4, control rats presented more homogeneous microbial communities than cirrhotic rats.

In order to determine the microbial taxon or group of taxa that is increased or decreased in the cirrhotic rats versus controls, we performed ANOVA. Clostridiales, a group of bacteria belonging to the Firmicutes phylum, were found to be 6.6-fold more abundant in controls than in cirrhotic rats ($p = 0.006$; FDR = 0.087; fig. 5a). Furthermore, an unknown species belonging to the *Janibacter* genus (Actinobacteria phylum) was found to be 2.5-fold more abundant in rats with cirrhosis (2.9% of the sequences) than in controls (1.2% of the sequences; $p = 0.019$; FDR = 0.37; fig. 5b).

Correlation with Immune Responses

In order to identify the possible association between microbial taxa and inflammation, we measured cytokines such as IL-6 and IL-10 in blood samples in all the 7 study rats. The concentration of these two cytokines, although higher in cirrhotic rats [492 (SD = 293) vs. 179 pg/ml (SD = 213), respectively] than in control rats [388 (SD = 100) vs. 47 pg/ml (SD = 35 pg/ml), respectively], was not significantly different between the two groups. However, using the Pearson correlation, we found that MLN bacteria belonging to the Actinobacteria phylum, and in particular *Bifidobacterium animalis,* were significantly and positively correlated with high IL-10 expression ($p =$

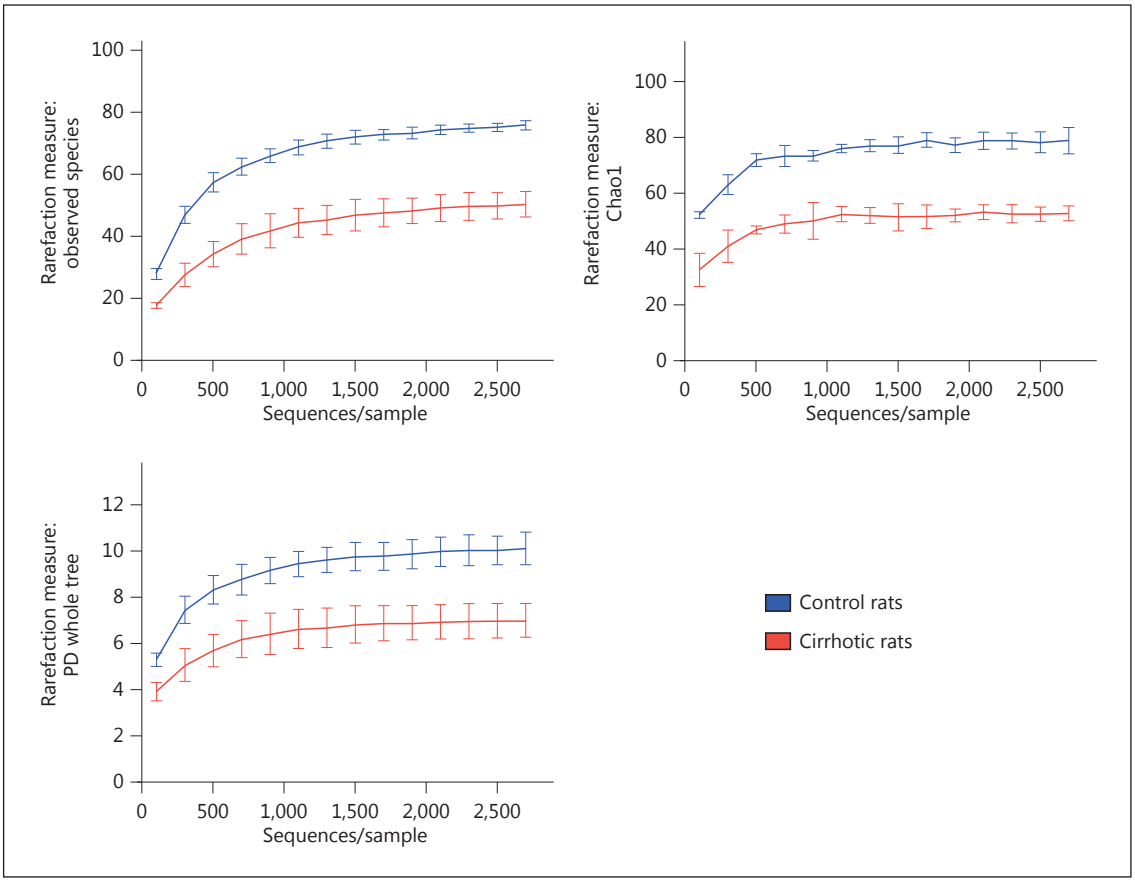

Fig. 2. Higher microbial richness in control rats. Control rats (n = 4) showed a higher microbial diversity ($p < 0.05$) than cirrhotic rats (n = 3) based on the 16S rRNA gene sequence analysis, as assessed by three richness metrics: number of observed species, Chao1 and phylogenetic diversity (PD).

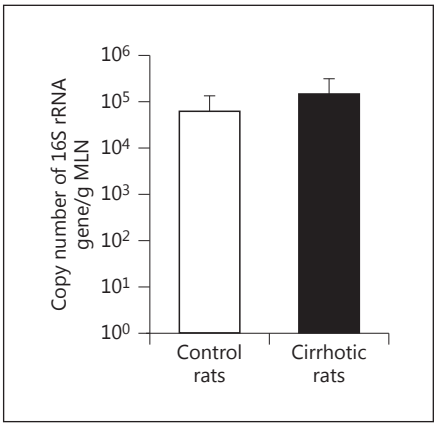

Fig. 3. Microbial load in MLNs of control and cirrhotic rats. Concentration of microbial DNA is similar in control (n = 4) and cirrhotic (n = 3) rats assessed by qPCR of the 16S rRNA gene. All reactions were performed in triplicate. The standard curve obtained from a 10-fold serial dilution of the template produced an R^2 value of 0.993 and a slope of –3.1.

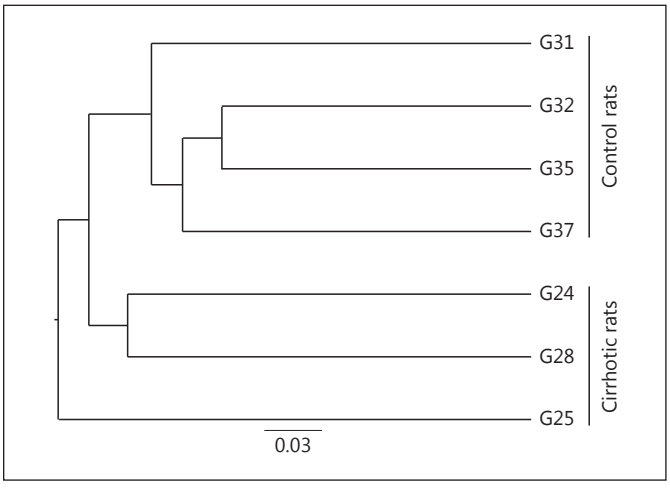

Fig. 4. Control and cirrhotic rats presented a distinct microbial composition. Two distinct clusters were identified using the UPGMA tree based on unweighted UniFrac analysis of 16S rRNA gene sequences. Scale bar represents 3% sequence divergence.

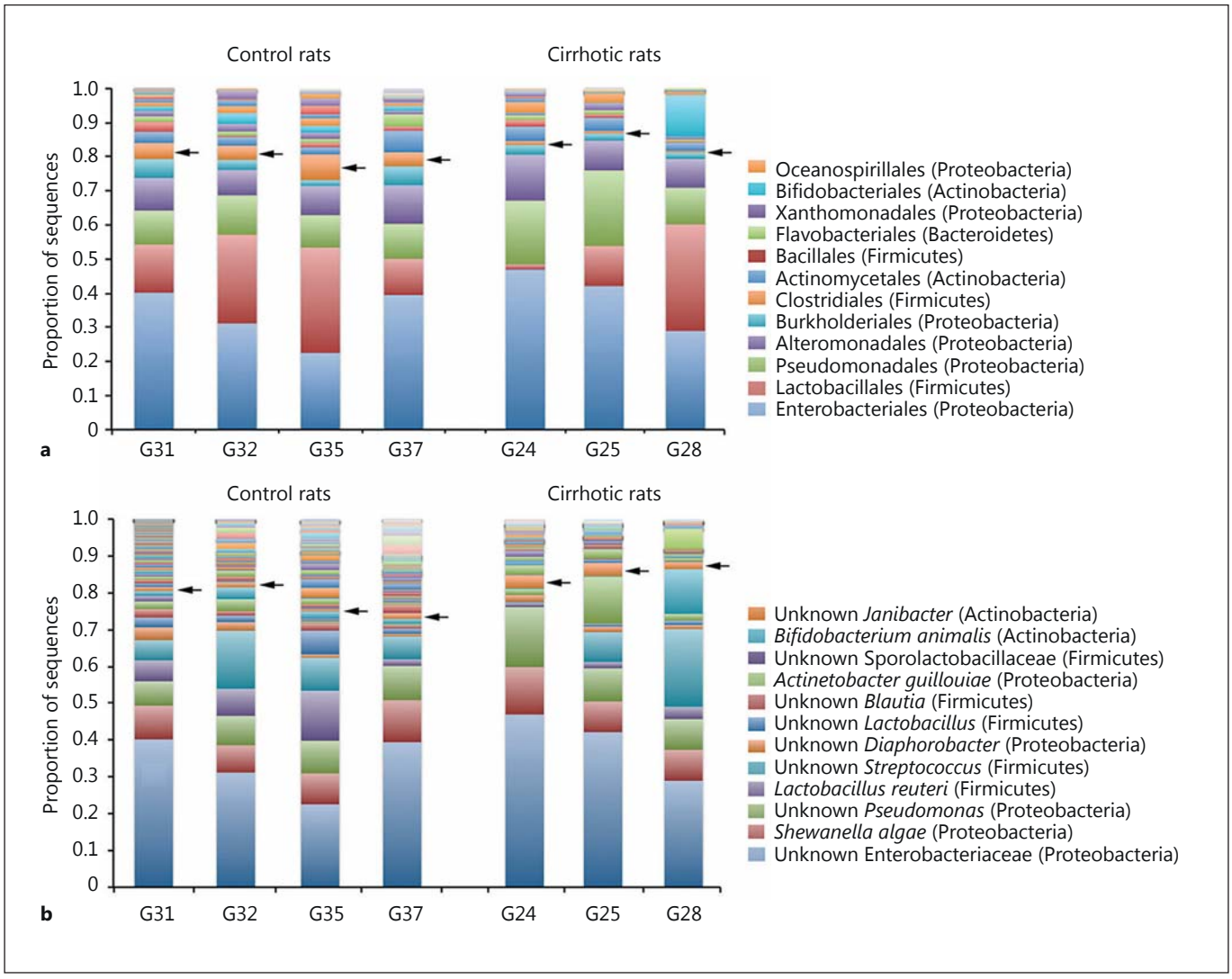

Fig. 5. Dysbiosis in MLNs of cirrhotic rats compared to control rats. **a** Control rats presented a higher proportion of Clostridiales (arrow, order level) than cirrhotic rats ($p = 0.006$; FDR = 0.087). **b** Cirrhotic rats showed a higher proportion of a taxon from the *Janibacter* genus (arrow, species level) than cirrhotic rats ($p = 0.014$; FDR = 0.27). Only taxa or order of taxa accounting for >1% of the sequences are represented in the legends.

0.002; FDR = 0.03; r = 0.94; fig. 6). However, we did not observe a significant correlation between a bacterium or a group of bacteria and IL-6 expression.

Discussion

To our knowledge, although it has been shown that more than 1 bacterial species is translocated into MLNs [14], our study is the first to report a high microbial diversity of bacterial DNA.

Our previous studies demonstrated that fecal microbiota of healthy rats is dominated by Firmicutes (74%) and Bacteroidetes (23%), whereas Actinobacteria and Proteobacteria are minor constituents [20]. However, in this study, our observation showed that MLNs from control and cirrhotic rats harbored a majority of Proteobacteria. This would suggest that bacteria from this phylum more than other phyla have a greater ability to attach and thus penetrate the mucosal barrier. Indeed, Clegg et al. [25] showed that Gram-negative bacteria such as *Escherichia coli* (Proteobacteria) produce functional adhesins

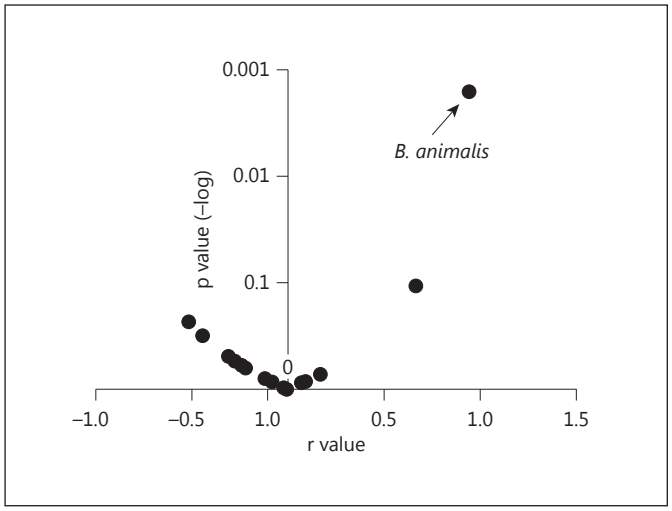

Fig. 6. Correlation between microbial taxa present in all MLNs and serum IL-10 concentrations using ANOVA. The analysis provided an r and a p value for each microbial taxon in the 7 rats. The plot illustrates the r value against the p value for each taxon (represented by a dot).

that play a role in bacterial-host cell interactions. Thus, more antigens from these bacteria would be presented to the immune cells in MLNs and would therefore play an important role in homeostasis. This hypothesis would explain cases where lower abundant intestinal phyla, such as Proteobacteria, have been found to be associated with conditions like irritable bowel syndrome, a low-grade inflammatory disorder [26, 27], inflammatory bowel disease [28] and cirrhosis [29, 30].

Previous studies using microbiological culture and conventional PCR to detect bacterial DNA have shown that bacterial translocation to MLNs is rare in control rats and occurs between 37 and 87% of the cirrhotic rats with ascites [9, 30–33]. Moreover, the bacterial translocation in these studies was mostly monomicrobial and less frequently bimicrobial. However, using pyrosequencing, our study suggests that the presence of polymicrobial DNA in MLNs is a physiological feature in control rats and that bacterial translocation should no longer be considered a dichotomic and monomicrobial event but instead as a constant and polymicrobial phenomenon. This notion is in agreement with a recent study, also using pyrosequencing, that found polymicrobial DNA in most sterile ascitic fluid samples of patients with cirrhosis [11]. In this setting, what would be relevant is not that bacterial DNA was detected in the MLNs but the quantitative and qualitative characteristics of such polymicrobial DNA. Moreover, we observed a decrease in Clostridiales in MLNs of cirrhotic rats. This is in line with the study of Gomez-Hurtado et al. [34] who reported lower *Clostridia* spp. in fecal samples in mice treated with CCl_4 compared to control animals, and this was associated with a proinflammatory scenario.

Our findings in MLNs reveal differences in the microbial community composition between cirrhotic and control rats and a lower microbial diversity without a decrease in microbial load in the former. The observation of lower diversity is a common trait associated with human disorders such as obesity, inflammatory bowel disease and colic in infants [35–37]. These observations support the notion of missing bacteria replaced by a few pathogenic ones in disease conditions.

Commensal bacteria provide defense against pathogenic ones, not simply by competing for nutrients and physical niches but also by inducing specific immune responses. Indeed, the development of the mucosal immune system is dependent on the type of bacteria that are present in the lumen and that can be captured by immune cells such as dendritic cells. Previous studies have reported that fecal *Bifidobacterium* is reduced in patients with cystic fibrosis or with hepatitis B virus-induced chronic liver disease [38, 39].

Investigating translocation of commensal bacteria using qPCR in a mouse model of type 2 diabetes, which is associated with systemic low-grade inflammation, Amar et al. [40] found high numbers of intestinal bacteria in the adipose tissue and blood. They demonstrated that the translocation event could be reverted by 6 weeks of treatment with the probiotic strain *B. animalis* subsp. *lactis* 420. Our finding, which showed a positive correlation between *B. animalis* and high anti-inflammatory cytokine levels in MLNs, is in line with this previous work.

Our study, although using a very high-throughput technique to study the MLN microbiome, presents several limitations. Indeed, considering that this work is a pilot and laborious study, we used a low number of rats. Furthermore, our study does not allow understanding whether the difference in MLNs is a consequence of the difference in the gut microbiome or a difference in selective translocation. In future studies, in order to be able to distinguish between these two possible causes, we could also consider analyzing the gut microbiome. Finally, our study does not distinguish the presence of viable from nonviable bacteria, which could be solved, in the future, using a PCR-based method using propidium monoazide [41].

In conclusion, this pilot study revealed the presence of high 16S rDNA diversity in MLNs of both control and

cirrhotic rats. The latter presented significantly lower bacterial diversity and a dysbiosis of the microbial community.

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Disclosure Statement

There is no conflict of interest.

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ANNEX 2. Publication related to the thesis. Santiago *et al*. 2014 “Processing faecal samples: a step forward for standards in microbial community analysis.”

Santiago et al. BMC Microbiology 2014, 14:112
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BMC Microbiology

RESEARCH ARTICLE Open Access

Processing faecal samples: a step forward for standards in microbial community analysis

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Abstract

Background: The microbial community analysis of stools requires optimised and standardised protocols for their collection, homogenisation, microbial disruption and nucleic acid extraction. Here we examined whether different layers of the stool are equally representative of the microbiome. We also studied the effect of stool water content, which typically increases in diarrhoeic samples, and of a microbial disruption method on DNA integrity and, therefore, on providing an unbiased microbial composition analysis.

Results: We collected faecal samples from healthy subjects and performed microbial composition analysis by pyrosequencing the V4 region of the 16S rRNA gene. To examine the effect of stool structure, we compared the inner and outer layers of the samples (N = 8). Both layers presented minor differences in microbial composition and abundance at the species level. These differences did not significantly bias the microbial community specific to an individual. To evaluate the effect of stool water content and bead-beating, we used various volumes of a water-based salt solution and beads of distinct weights before nucleic acid extraction (N = 4). The different proportions of water did not affect the UniFrac-based clustering of samples from the same subject However, the use or omission of a bead-beating step produced different proportions of Gram-positive and Gram-negative bacteria and significant changes in the UniFrac-based clustering of the samples.

Conclusion: The degree of hydration and homogenisation of faecal samples do not significantly alter their microbial community composition. However, the use of bead-beating is critical for the proper detection of Gram-positive bacteria such as *Blautia* and *Bifidobacterium*.

Keywords: 16S ribosomal RNA, Faecal sample collection, Needs for standardisation, Diarrhoea, Bead-beating

Background

In the 1680s, Anton van Leeuwenhoek used homemade microscopes to provide the first description of faecal bacteria. Faecal specimens contain one of the densest microbial communities known, they have been shown to contain similar microbial community than the colon [1] and do not require an invasive collection protocol. Therefore they continue to be the samples most widely used for studying the intestinal microbiome, a collection of microbial genomes. In the last ten years, the greatest insights into the human intestinal microbiome have come about as a result of the application of metagenomics approaches to faecal samples, as attested by more than 1294 scientific publications found under the terms “human faecal microbiome” and “human fecal microbiome ” in PubMed.

Metagenomics approaches in biomedicine seek to provide a comprehensive picture of the diversity and abundance of dominant and subdominant microbial species in health [2,3] and in diseased states such as inflammatory bowel disorders (IBDs), irritable bowel syndrome (IBS) and other functional bowel disorders (FBD) [4-7]. During the course of these diseases, stool consistency is altered, varying from very hard (in constipation) to entirely liquid (in diarrhoea), as determined by the Bristol stool scale [8].

Diarrhoea is defined as an abnormally frequent discharge of semi-solid or fluid faecal matter from the bowel. As such, it usually implies a large percentage of water. A

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normal stool sample is considered to have a water content of about 75%, while that of a diarrhoeic stool is > 85% [9]. The freezing of specimens containing water causes the formation of ice crystals, which damage the microbial cell wall. Consequently, there is an increased release of cellular components such as DNase and RNase, which in turn may degrade nucleic acids at the beginning of the DNA extraction procedure. In intestinal disorders, such as IBD, IBS, and infectious diseases, the sampling of diarrhoeic stools is common [10,11]. However, how the water content of these samples affects the integrity of microbial DNA, and therefore the analysis of microbial composition, is unclear.

Steps such as stool homogenisation during collection or mechanical cell wall breaking during DNA extraction may affect the analysis of the microbial community. To date, no study on stool homogenisation or mechanical cell wall breaking using high-throughput sequencing technique has been reported. An appropriate collection protocol, together with a better understanding of the characteristics of a stool, is critical for downstream microbial community analysis.

Here we tested various factors that may affect microbial community analysis during stool sample collection and DNA extraction steps using gel electrophoresis and pyrosequencing of the 16S rRNA gene. In this regard, we examined the effect of homogenising the stool before freezing, the addition of a physiological solution to the stools to simulate a diarrhoeic condition before freezing, and the use of beads to breakdown the microbial cell wall during DNA extraction.

Results and discussion

Experimental design

Faecal samples were collected from healthy volunteers (n = 8) who had not taken antibiotics during the previous three months. Fresh samples were aliquoted as described below.

To test whether different layers of a stool sample unequally represent the microbiome, we compared the microbial composition of each faecal sample in three conditions: fully homogenised during sample collection, non-homogenised outer layers, and non-homogenised inner layers. For this comparison, two aliquots from each volunteer (#1 to #8, named L1 to L8) and for each condition were used. Thus, a total of 48 samples were prepared for microbial composition analysis.

To evaluate the effect of stool water content and the bead-beating technique on the integrity of microbial DNA and, therefore, on microbial composition analysis, fresh stool samples were homogenised with an increasing proportion of phosphate-buffered saline (PBS), as indicated in Table 1. Assuming that a normal stool contains 75% (range 56.6%–84.9%) of water, the dilutions tested corresponded to 75%, 80%, 87.5%, 93.8%, 97.5% and 99.5% of water content, respectively, which reflect the range of typical diarrhoeic samples [9,12]. Similar amounts of each diluted sample were then disrupted with and without a bead-beating step. This procedure was carried out for four of the eight volunteers cited above (#1, #3, #5 and #8, named DL1, DL3, DL5 and DL8). Thus, a total of 46 samples were collected for microbiome analysis.

Table 1 Addition of PBS to obtain stools with a range of water content

ID	Weight (mg)	Presence of beads	PBS (μl)	Water content
L#	250	yes	-	75.0%
DL#.00	250	yes	0	75.0%
DL#.25	187.5	yes	62.5	80.0%
DL#.50	125	yes	125.0	87.5%
DL#.75	62.5	yes	187.5	93.8%
DL#.90	25	yes	225.0	97.5%
DL#.98	5	yes	245.0	99.5%
DL#B.00	250	yes	-	75.0%
DL#B.25	187.5	yes	-	75.0%
DL#B.50	125	yes	-	75.0%
DL#B.75	62.5	yes	-	75.0%
DL#B.90	25	yes	-	75.0%
DL#B.98	5	yes	-	75.0%
DL#P.50	125	-	125.0	87.5%
DL#P.75	62.5	-	187.5	93.8%
DL#P.90	25	-	225.0	97.5%
DL#P.98	5	-	245.0	99.5%
DL#C.50	125	-	-	75.0%
DL#C.75	62.5	-	-	75.0%
DL#C.90	25	-	-	75.0%
DL#C.98	5	-	-	75.0%

# indicates the identification number for each subject.
L# = stands for layer in the homogenisation study.
DL# = the "D" stands for diarrhoea in the water content study; the "L" refers to samples that have been also used in the homogenisation study, that contained PBS and underwent a bead-beating step.
DL#B = samples that did not contain PBS but underwent a bead-beating step.
DL#P = samples that contained PBS but did not undergo a bead-beating step.
DL#C = samples that did not contain PBS and did not undergo a bead-beating step.

Effect of stool homogenisation during collection

Usually, participants are instructed to homogenise their stool samples during collection. However, given the laborious and unpleasant nature of this task, it is possible that they might not have fully complied with this procedure. To evaluate the impact of homogenisation on the composition of the microbial community, we analysed the 48 samples as specified in the experimental design cited above (L#) by means of pyrosequencing the 16S rRNA gene at a normalised depth of 6173 sequences of 290 bp per sample.

The microbial profile at the species level was quite similar between a portion of the stool collected in the outer area, in the inner area and after homogenisation, except for sample LO4.1, which showed a similar diversity but distinct abundance of Operational Taxonomic Units (OTUs) (Figure 1, Additional file 1: Table S1). This observation was confirmed by an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering analysis based on unweighted and weighted UniFrac distances (Figure 2). Sample LO4.1 from subject #4 was the only one that clustered far from the other samples from the same stool when both microbial composition and abundance were considered (weighted UniFrac analysis, Figure 2B).

Effect of stool water content

To evaluate how stool water content affects the microbial community, we analysed the 46 samples from four out of the eight participants, as described in the experimental design section above.

After the extraction procedure, genomic DNA was loaded in an Agilent 2100 Bioanalyzer chip in order to evaluate integrity. A comparison of the DNA extracted from DL1 samples (presence of beads and PBS) with those of DL1B's (presence of beads but not PBS) showed that the addition of PBS caused greater genomic DNA degradation (Figure 3A). This finding was confirmed by a decrease in DNA size to lower than 10 Mbp with 125 mg of stool (sample DL1.50, Table 1) and 50% PBS. In contrast, in the absence of PBS this degradation was also observed but only when the stool weighed 62.5 mg (DL1B.75). Interestingly, we observed a double effect of stool water content and bead-beating when dealing with a small amount of stool matter.

Although the presence of PBS could increase the degradation of genomic DNA, the microbial community profile was not affected at the species level (Figure 3B). This observation could be explained by the fact that the microbial analysis was based on the PCR amplification of the V4 region of the 16S rRNA gene, which is around 300 bp, whereas the degraded genomic DNA fragments were larger than 3000 bp. Moreover, this size may be sufficient for shotgun sequencing as DNA would be cut into fragments of between 400 and 800 bp. However, further sequencing experiments are required to confirm that the gene content analysis is not biased.

Effect of bead-beating during DNA extraction

A bead-beating step during DNA extraction is required to break down the cell wall of Gram-positive bacteria [13]. To evaluate the effect of bead-beating on the microbial community of diarrhoeic samples, we compared conditions

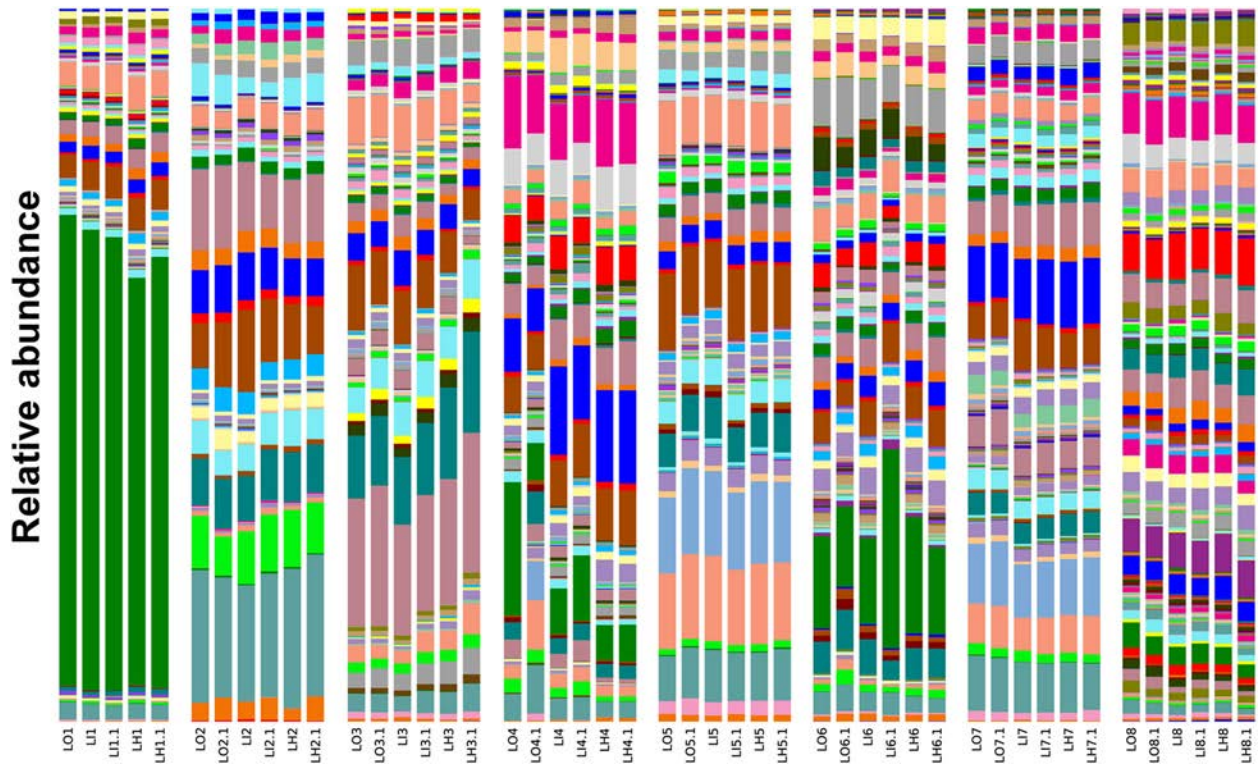

Figure 1 Spatial organization of the microbial community (species level) in stool specimens. 250 mg of stool (N = 8) was collected in the outer (LO) and inner area (LI) layer and once the stool had been homogenised (LH). Stools were collected in duplicates for each condition.

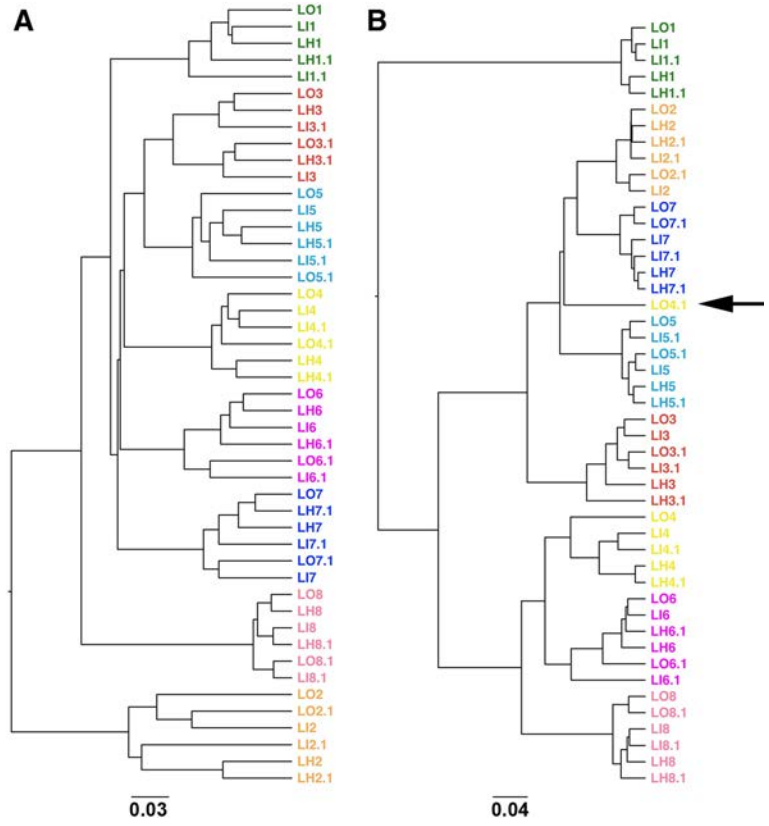

Figure 2 UPGMA clustering based on weighted (A) and unweighted UniFrac (B) distance analysis. 250 mg of stool (N = 8) was collected from the outer (LO) and inner (LI) layers and after the stool had been homogenised (LH). Stools were collected in duplicates for each condition (48 samples in total). Unweighted UniFrac allows clustering by taking into account only the microbial composition, while weighted UniFrac considers both composition and abundance of OTUs.

with and without a bead-beating step, and with and without an increasing volume of PBS (samples DL5 and DL8 versus DL5P and DL8P). Although the disruption step caused degradation of genomic DNA, in an increased volume of PBS, it did not greatly modify the microbial community profile (Figure 4B). Moreover, samples containing a different volume of PBS (see samples DL5.00 to DL5.98 and DL8.00 to DL8.98) clustered together (Figure 5A and B), as shown by an UPGMA-UniFrac analysis, and presented a similar alpha diversity, as measured by phylogenetic diversity (PD) metric (Additional file 2: Figure S1). However, in the absence of bead-beating during the extraction procedure, genomic DNA did not show any sign of degradation at any volume of PBS tested, but the DNA yields were lower than with bead-beating (the average sum was 816 ng/μl versus 941 ng/μl with bead-beating). The microbial profile of these samples also differed completely to that of those subjected to bead-beating (DL# versus DL#P and DL#C; where # = 5 or 8). As expected, the absence of bead-beating significantly decreased the detection of Gram-positive bacteria such as Firmicutes and Actinobacteria phyla (Figure 4B). At the genus level, proportions of *Blautia* and *Bifidobacterium* were decreased by at least 5- and 14-fold, respectively (Mann Whitney test, $p < 0.001$) (Figure 5).

The UPGMA clustering analysis based on the unweighted UniFrac method, which takes into account the microbial composition, did not show separation of the samples with or without a bead-beating step (Figure 6A). However, when the analysis was based on a weighted UniFrac method, which considers both microbial composition and abundance, samples from one of the four subjects clustered separately (Figure 6B). Here we show that the inclusion of this procedure dramatically changed both the migration profile of the genomic DNA and the taxonomic profile of stool samples.

Conclusion

Microbial community studies involve a variety of procedures, ranging from sample collection to sequence data interpretations. Given the increasing relevance of metagenomics for research into intestinal disorders, it is crucial that the data generated in each study be optimally comparable across all those already underway. However, strong biases can be introduced into stool research, in

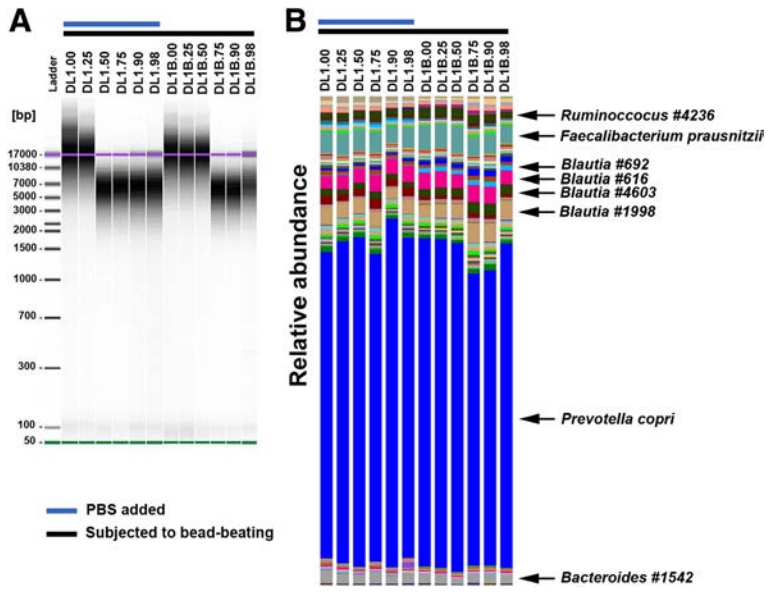

Figure 3 Effect of water content on genomic DNA integrity. (A) Gel electrophoresis analysis. For each sample, genomic DNA equivalent to 1 mg of faecal sample was loaded on an Agilent 2100 Bioanalyzer chip using the Agilent 12000 kit. DL1 corresponds to participant L1 from the homogenisation evaluation. **(B)** Microbial diversity at the species level. The taxonomic analysis was performed using a cut-off of 97% similarity. The "#" followed by a number indicates an arbitrary identifier for an unknown OTUs.

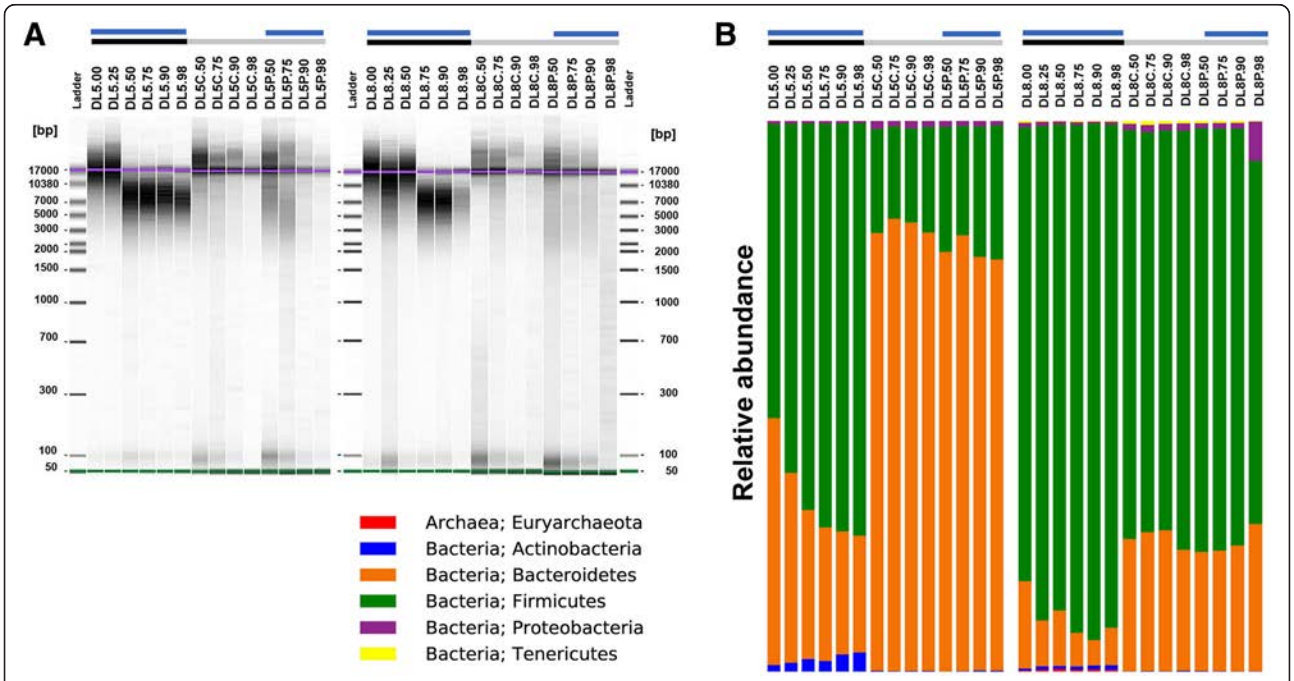

Figure 4 Effect of bead-beating on genomic DNA integrity and on microbial community composition. (A) Gel electrophoresis analysis. For each sample, genomic DNA equivalent to 1 mg of faecal sample was loaded on an Agilent 2100 Bioanalyzer chip using the Agilent 12000 kit. **(B)** Microbial diversity profile at the phylum level. Sample identification is identical to that indicated in the legend of Figure 3. DL5 and DL8 correspond to the participants L5 and L8 from the homogenisation evaluation. Samples with the identification starting with DL5C and DL8C were not subjected to bead-beating nor did they contain PBS. DL5P and DL8P contained only PBS. Black bars indicate the samples subjected to bead-beating and grey bars those that were not, while blue bars show the samples to which PBS was added.

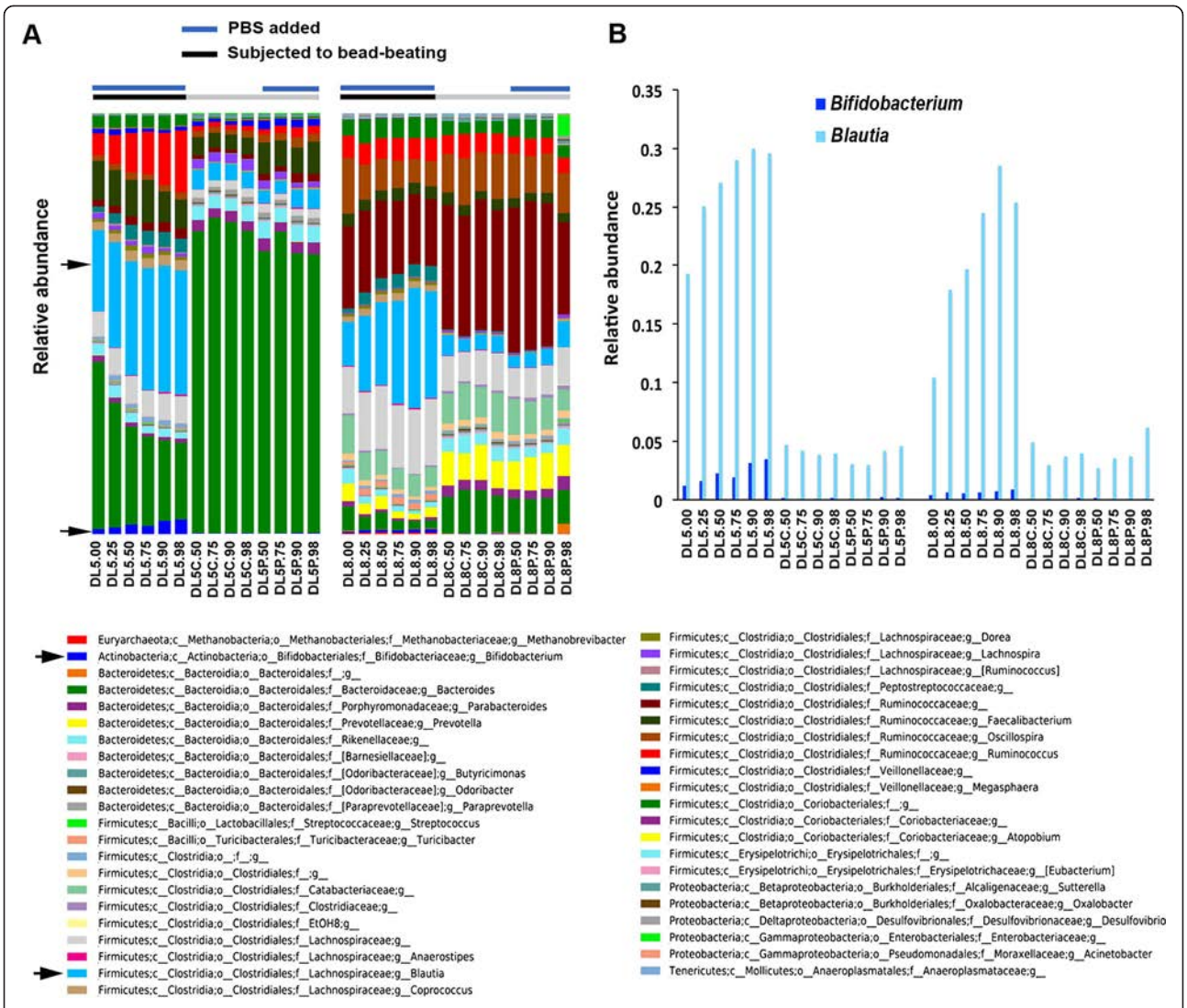

Figure 5 Microbial profile at the genus level. (A). All OTUs are shown. The arrows indicate the detection of *Bifidobacterium* and increase in the detection of *Blautia* with the bead-beating procedure. Black bars represent the samples subjected to bead-beating and grey bars those that were not, while the blue bars indicate the samples to which PBS was added. **(B)**. Relative abundance of *Blautia* and *Bifidobacterium*. The identification of the samples is identical to that shown in the legend of Figure 3. DL5 and DL8 correspond to participants L5 and L8 from the homogenisation evaluation. Samples DL5C and DL8C represent those that were not submitted to bead-beating nor did they contain PBS. DL5P and DL8P contained only PBS.

particular during stool collection and storage and during DNA extraction. We previously recommended that stool samples be kept at room temperature and be brought to the laboratory within 24 h after collection or alternatively be stored immediately at −20°C by the volunteer in a home freezer, to be later transported in a freezer-pack to the laboratory, where all samples are stored at −80°C before further treatment [14].

Our findings from the present study indicate that homogenisation of the stool during collection is recommendable but not indispensable. Indeed, samples collected from the inner and outer layers of stool samples showed a similar microbial composition and abundance. Moreover, we show that the percentage of water typically found in diarrhoeic samples does not affect the clustering of samples from the same subjects. To validate our results, analysis of diarrhoeic samples could be compared with non-diarrhoeic ones from the same individual; however, the collection of these two types of samples from the same healthy subjects would be complicated for ethical reasons. Moreover, since other next generation sequencing platforms will allow a greater sequencing depth, this may allow a deeper characterization of the microbial community and could reveal additional differences in the microbial community composition for the various conditions measured in this study.

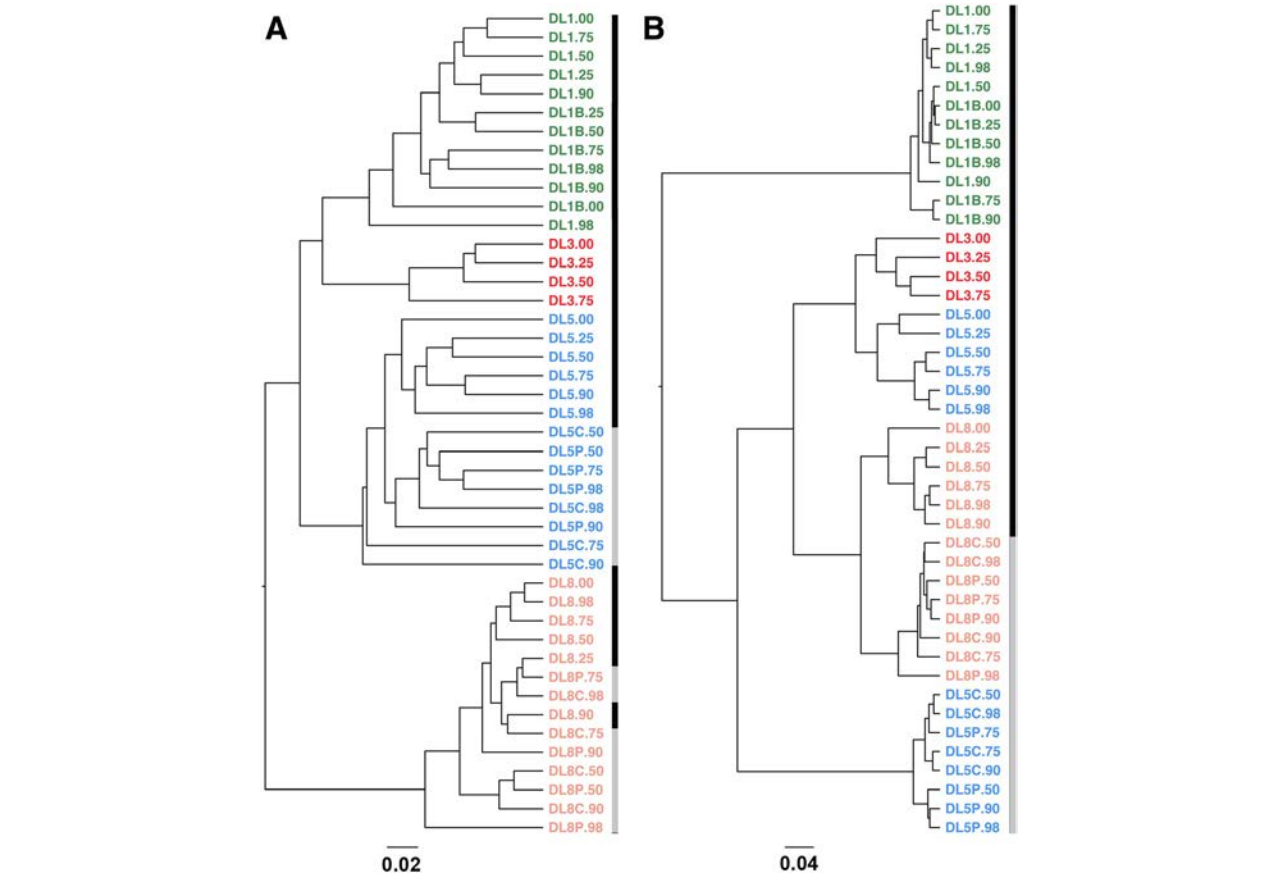

Figure 6 UPGMA clustering based on weighted (A) and unweighted UniFrac (B) distance analysis. Unweighted UniFrac allows the clustering of samples by taking into account only the microbial composition, whereas weighted UniFrac considers both composition and abundance of OTUs. Black bars also indicate the samples subjected to bead-beating and grey bars those that were not.

Finally, our study also reveals that microbial disruption by bead-beating allows greater detection of Gram-positive bacteria such as *Blautia* (Firmicutes phylum) and *Bifidobacterium* (Actinobacteria phylum), commonly detected in human stools. In conclusion, the hydration of faecal samples and their degree of homogenisation do not significantly alter their microbial community composition and structure. However, although the mechanical disruption of microbial cells causes genomic DNA degradation in simulated diarrhoeic stool samples, our findings confirm that this step is necessary for the detection of Gram-positive bacteria such as *Blautia* and *Bifidobacterium*.

Methods

Ethics statement

Subjects provided their written consent to participate in this study, and the Institutional Review Board of the Vall d'Hebron Hospital (Barcelona, Spain) approved this consent procedure.

Sample collection protocol

Stools were collected from eight healthy participants. The collection protocol involved providing participants with an ice bag containing an emesis basin (Ref. 104AA200, PRIM S.A, Spain), a 50-mL sterile sampling bottle (Ref. 409526.1, Deltalab, Spain), a sterile spatula (Ref. 441142.2, Deltalab, Spain), and gloves (Additional file 3: Figure S2) during their visit to the laboratory. For the purpose of stool collection, the participants were instructed to do the following once at home: 1) use the emesis basin to collect the stool; 2) after the deposit, transfer it to the sampling bottle ensuring no homogenisation; 3) take it to the lab within the first 3 hours after deposit; and 4) in the laboratory, the samples were processed as mentioned in the experimental design, and then the samples were stored at −80°C.

Naming convention

Since the samples from same individuals were used to test different factors that could affect microbial composition, a

labeling nomenclature had to be settled down as indicated in Table 1. The "D" stands for "diarrhoea" in the water content study. The "L" stands for "layer", "O" for "outer" and "I" for the "inner" layer of the stool, and "H" for "homogenised stool" in the homogenisation evaluation. The "P" stands for samples that contained PBS to simulate diarrhoea not undergoing bead-beating, while "B" stands for samples that did not contain PBS, but underwent bead-beating. Samples with the "C" label are controls that did not contain PBS and did not undergo bead-beating. The numbers 1–8 signify the 8 different volunteers.

Genomic DNA extraction

To evaluate the need for stool homogenisation during collection, aliquots (250 mg) of each sample were suspended in 0.1 M Tris (pH 7.5), 250 μl of 4 M guanidine thiocyanate, 40 μl of 10% N-lauroyl sarcosine and 500 μl of 5% N-lauroyl sarcosine, as previously described in [15]. DNA extraction was carried out by mechanical disruption of the microbial cell wall using beads (Lysing matrix E, MP Biomedicals, Spain). The disruption was performed by shaking the mixture using the Bead-Beater-8 (BioSpec, USA) at a medium speed of about 1500 oscillations/min for 3 minutes, followed by 3 minutes in ice and again followed by 5 minutes at a medium speed of about 1500 oscillations/min. Finally, nucleic acids were recovered from clear lysates by alcohol precipitation.

To evaluate the effect of stool water content and a bead-beating step, aliquots of samples were homogenised with various volumes of PBS (final weight of 250 mg) and with or without beads, as described in Table 1. They were then processed the same way as described above. In samples in which beads were not used, the bead-beater step was also omitted.

After genomic DNA extraction, an equivalent of 1 mg of each sample was used for DNA quantification using a NanoDrop ND-1000 Spectrophotometer (Nucliber). DNA integrity was examined by microcapillary electrophoresis using an Agilent 2100 Bioanalyzer with the DNA 12000 kit, which resolves the distribution of double-stranded DNA fragments up to 17,000 bp in length.

Microbial community analyses

454 pyrosequencing of the V4 variable region of the 16 S rRNA gene

To analyse bacterial composition, we subjected extracted genomic DNA to PCR-amplification of the V4 hypervariable region of the 16S rRNA gene. On the basis of our analysis done using PrimerProspector software [16], the V4 primer pairs used in this study were expected to amplify almost 100% of the Archaea and Bacteria domains. The 5' ends of the forward primer V4F_517_17 (5′-GC CAGCAGCCGCGGTAA-3′) [17] and the reverse primer V4R_805_19 (5′-GACTACCAGGGTATCTAAT-3′) [18] were tagged with specific sequences for pyrosequencing as follows: 5′-CCATCTCATCCCTGCGTGTCTCCGACTC AG-{MID}-{GCCAGCAGCCGCGGTAA}-3′ and 5′ CCT ATCCCCTGTGTGCCTTGGCAGTCTCAG-{GACTACC AGGGTATCTAAT}-3′. Tag pyrosequencing was performed using multiplex identifiers (MIDs) (Roche Diagnostics) of 10 bases, which were specified upstream of the forward primer sequence (V4F_517_17). Standard PCR amplification was run in a Mastercycler gradient (Eppendorf) at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 20 sec, 72°C for 40 sec, and a final cycle of 72°C for 7 min. PCR products were purified using a PCR Purification kit (Qiagen, Spain) and subsequently sequenced on a 454 Life Sciences (Roche) FLX system (Scientific and Technical Support Unit, Vall d'Hebron Research Institute, Barcelona, Spain), following standard 454 platform protocols.

16S rRNA sequence data analysis

A total of 1.47 million sequence reads from 96 samples were analysed using the default settings in the Quantitative Insights Into Microbial Ecology (QIIME) package of software tools [19]. The 16S rRNA sequences were quality-filtered and demultiplexed. These reads had an average length of 290 bp. Using the pick-otus protocol, we classified the sequence reads into OTUs on the basis of sequence similarity. Sequence reads were then clustered against the February 2011 release of the Greengenes 97% reference dataset (http://greengenes.secondgenome.com) [20,21]. Taxonomy was assigned using the Basic Local Alignment Search Tool (BLAST) [22]. The representative sequences of all OTUs were then aligned to the Greengenes reference alignment using PyNAST [18], and this alignment was used to construct a phylogenetic tree using FastTree [23] within QIIME. The resulting tree topology with associated branch lengths was used for subsequent diversity analyses (for many downstream analyses, samples were rarefied at 6173 and 9390 sequences per sample for the homogenisation and for the water content evaluations, respectively). One sample (LO1.1) was removed from the analysis because of low count reads. Alpha diversity was estimated using the phylogenetic diversity metric. Beta diversity analysis was performed using the UPGMA clustering method based on weighted and unweighted UniFrac distances [24].

Availability of supporting data

Sequences have been deposited in NCBI database with the accession number SRP040438.

Additional files

Additional file 1: Table S1. Legend of Figure 1.

Additional file 2: Figure S1. Alpha-diversity curves at a number of rarefaction depths. Each line represents the results of the alpha-diversity phylogenetic diversity whole tree metric (PD whole tree in QIIME) for all samples from subjects #5 and #8.

Additional file 3: Figure S2. Kit for stool collection (see the method section).

Abbreviations

PBS: Phosphate-buffered saline; UPGMA: Unweighted pair group method with arithmetic mean; QIIME: Quantitative insights into microbial ecology; OTU: Operational taxonomic units; BLAST: Basic local alignment search tool.

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

CM, AS and SP conceived and designed the study and drafted the manuscript. AS, SP and GM carried out the experiments. CM and XM did the 16S data generation and analysis. FA, JD and FG participated in design and coordination of the project. All authors read and approved the final manuscript.

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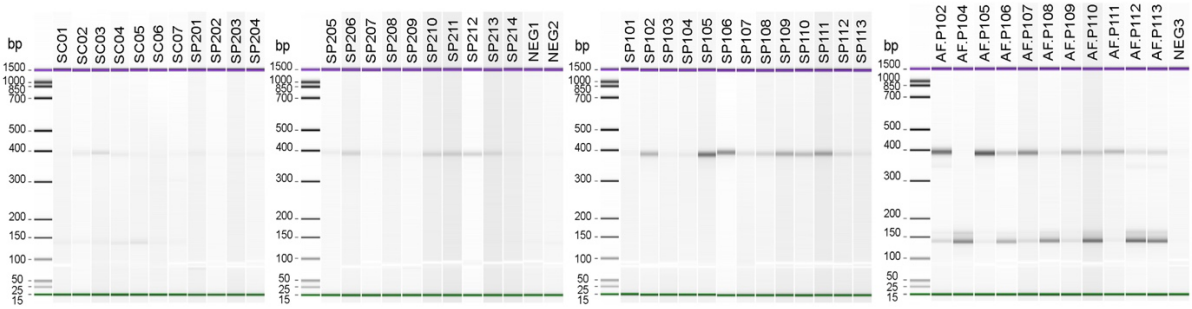
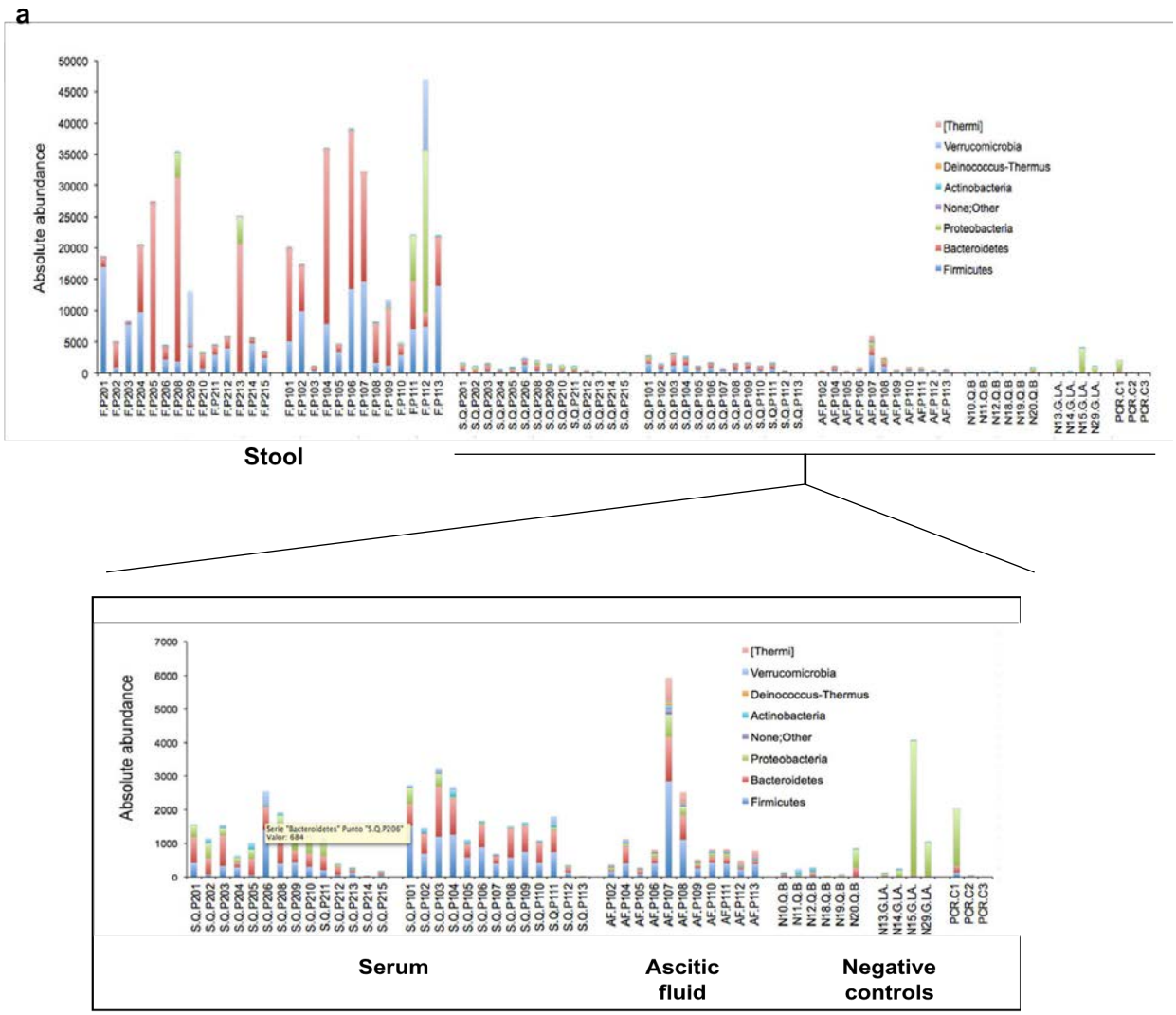
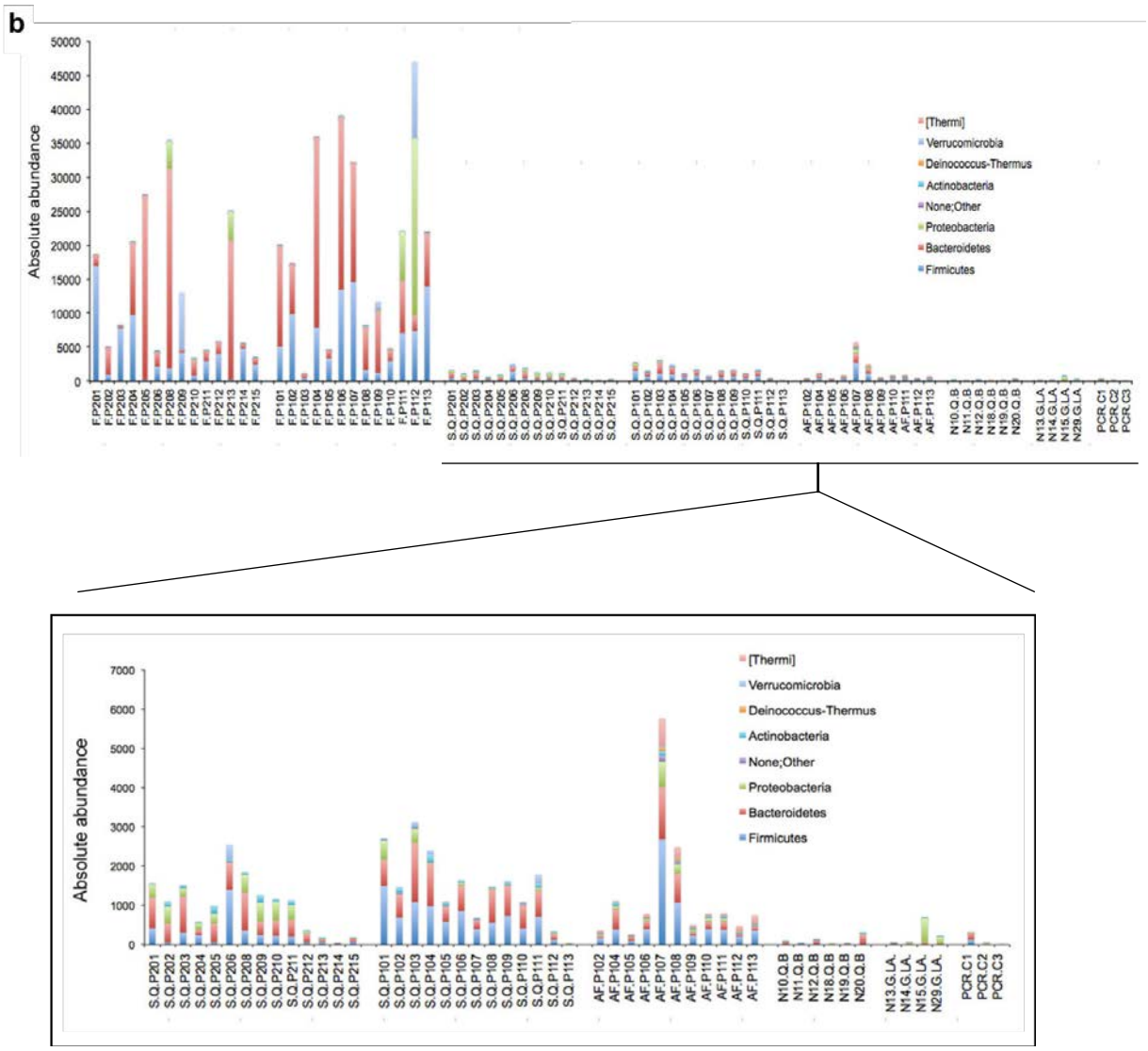
Cite this article as: Santiago *et al.*: Processing faecal samples: a step forward for standards in microbial community analysis. *BMC Microbiology* 2014 **14**:112.

ANNEX 3. SUPPLEMENTARY MATERIAL FROM CHAPTER 1

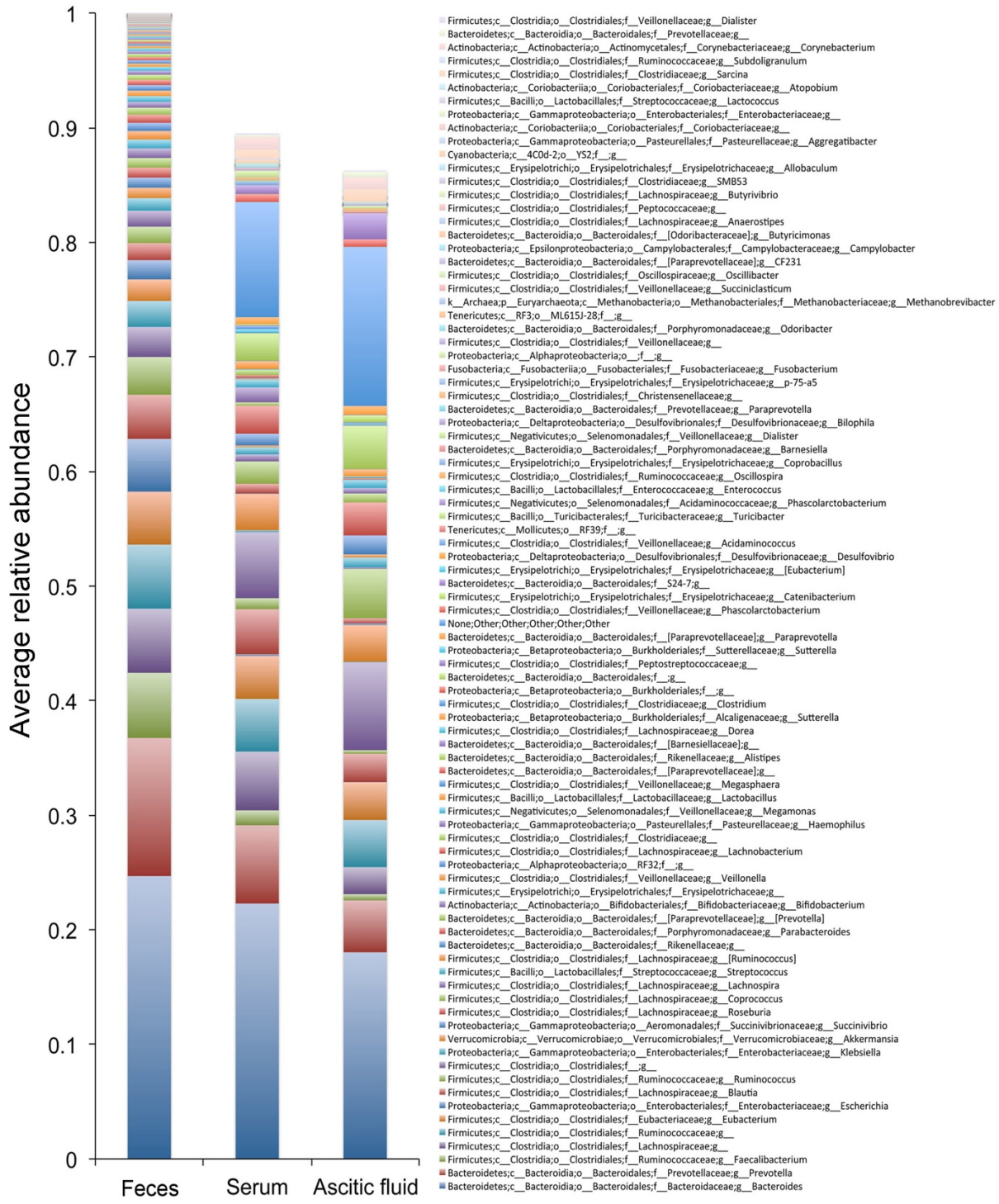


Supplementary Figure 1. Analysis of the PCR amplifications of the V4 region of the 16S rRNA gene on an electrophoretic gel. The products of the PCR amplification were loaded on an Agilent 2100 Bioanalyzer chip using the Agilent 1000 kit. The presence of the 16S gene amplicon can be detected at around 400 bp. The presence of DNA bands at around 150 bp can be identified as the primer dimers that are removed during sequence analysis.

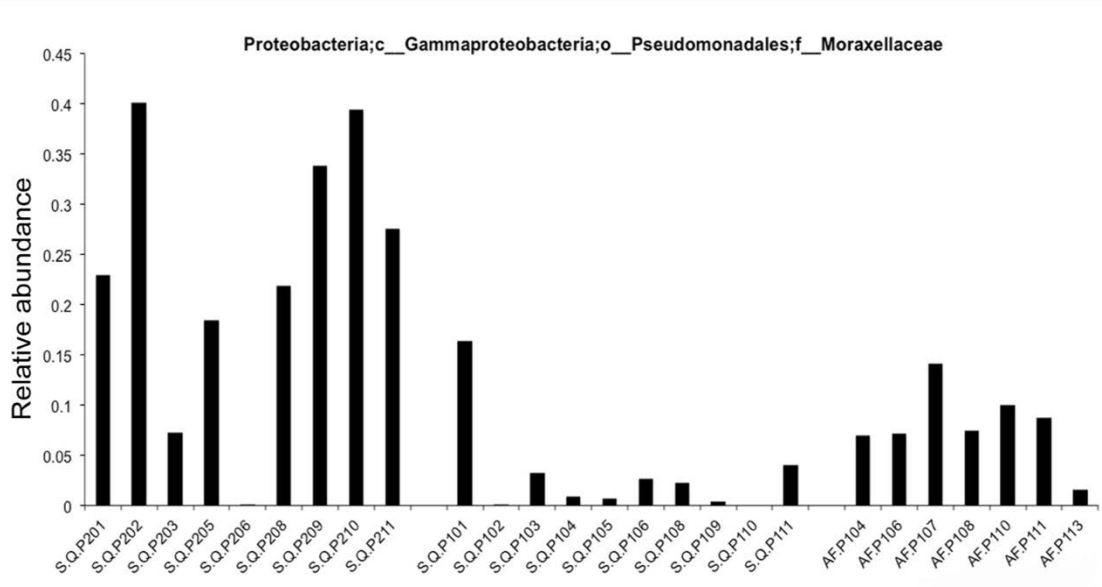




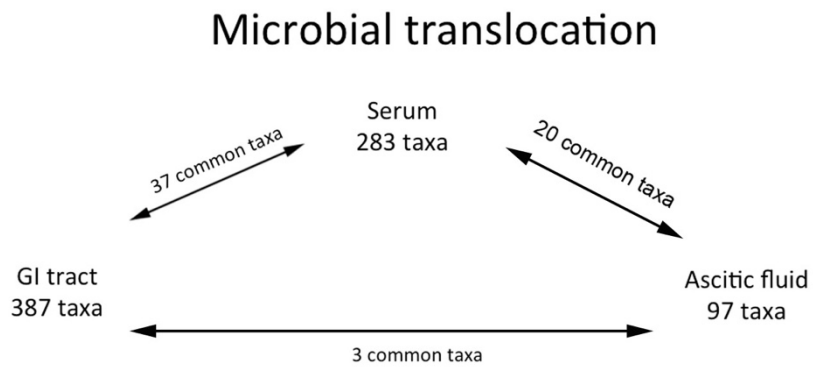
Supplementary Figure 2. Taxonomic profiling at the phylum level. (**a**) before filtering out contaminant sequences. (**b**) and after filtering out contaminant sequences. F = fecal sample; S = serum; AF = ascitic fluid; P = patient. Abundance of the phyla is given as absolute values.



Supplementary Figure 3. Taxonomic profiling of the 3 types of samples: feces, serum and ascitic fluid.



Supplementary Figure 4. Higher relative abundance of Moraxellaceae in serum of patients without ascites and ascitic fluid samples than in serum of patients with ascites. Analyses were performed on 16S rRNA V4 region data, rarefied to a depth of 700 reads per sample.



Supplementary Figure 5. Microbial translocation. The average number of taxa is indicated under each sample type. The average number of taxa common to two sample types is indicated between sample types.

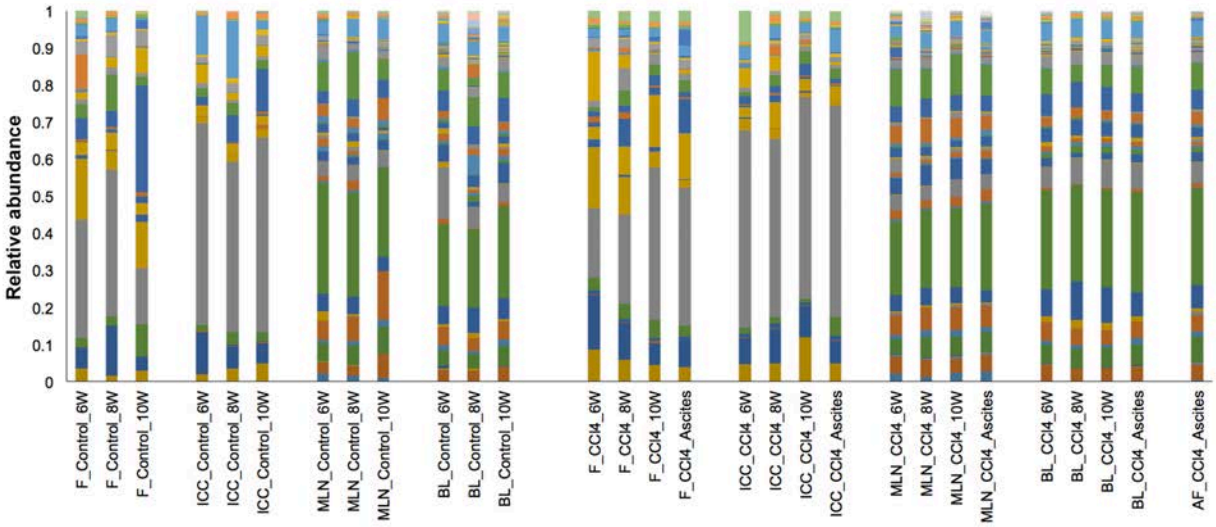
Supplementary Table 1. Rarefaction depths for sequences analyzed for each sample type.

	Total	Mean	Minimum	Maximum	Rarefaction depth
Faeces	2,008,492	45,647	19,930	131,334	19,930
Serum	117,322	4,345	204	8,497	1,000
Ascitic fluid	24,359	2,214	381	8,930	1,000

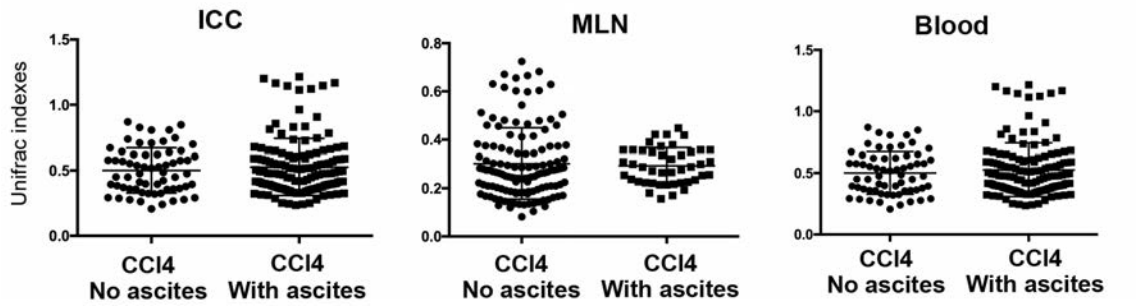
Supplementary Table 2. Clinical and analytical characteristics of patients in each group (with and without ascites). Results expressed as mean ± SEM or number of patients (%).

	Ascites (n = 13)	No ascites (n = 14)	p
Age (years)	68.9±3.0	67.3±2.3	0.69
Gender (male/female)	9 (69%)/4 (31%)	10 (71%)/4 (28%)	1.00
Body mass index (kg/m^2)	28.0±0.9	28.5±0.6	0.49
Diabetes	6 (46%)	4 (27%)	0.44
Child-Pugh A/B/C	3 (23%)/9 (69%)/1 (8%)	9 (64%)/4 (28%)/ 1(7%)	0.08
Child-Pugh score	7.7±0.4	6.1±0.4	**0.008**
MELD score	11.6±0.9	9.5±1.0	0.08
Etiology (alcohol/virus/alcohol+ virus/other)	7 (54%)/3 (23%)/1 (8%) 2 (15%)	9 (64%)/2 (14%)/ 0 (0%)/ 3 (21%)	0.49
Previous decompensation of cirrhosis	13 (100%)	9 (64%)	**0.04**
Previous ascites	13 (100%)	9 (64%)	**0.04**
Previous encephalopathy	1 (8%)	4 (29%)	0.32
Previous variceal bleeding	2 (15%)	0 (0%)	0.22
Beta-blockers	3 (23%)	6 (43%)	0.42
Diuretics	9 (69%)	7 (50%)	0.44
Proton pump inhibitors	3 (23%)	7 (50%)	0.23

ANNEX 4. SUPPLEMENTARY MATERIAL FROM CHAPTER 2

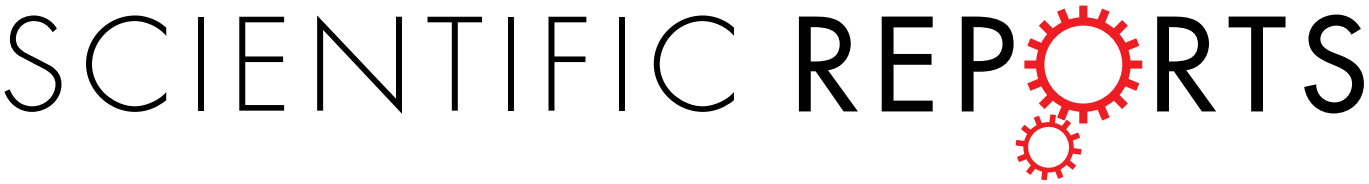


Supplementary Figure 1. Temporal and spatial distribution of the microbiome at the genus level.



Supplementary Figure 2. Microbiome instability. Weighted UniFrac distances were calculated between different time periods (weeks 6, 8, 10) for rats producing ascites and rats without ascites. UniFrac indexes obtained at week 6 were compared to those at weeks 8 and 10 or at time of ascites production. Progression of cirrhosis to ascites was not associated with instability of the microbiome composition in ICC nor in MLN ($p > 0.34$; Mann Whitney test), but in blood samples ($p = 0.04$; Mann Whitney test).

ANNEX 5. Publication related to the thesis. Sanchez et al. 2017 “Fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 reduces bacterial translocation in rats treated with carbon tetrachloride.”



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Fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 reduces bacterial translocation in rats treated with carbon tetrachloride

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Probiotics can prevent pathological bacterial translocation by modulating intestinal microbiota and improving the gut barrier. The aim was to evaluate the effect of a fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 on bacterial translocation in rats with carbon tetrachloride (CCl_4)-induced cirrhosis. Sprague-Dawley rats treated with CCl_4 were randomized into a probiotic group that received fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 in drinking water or a water group that received water only. Laparotomy was performed one week after ascites development. We evaluated bacterial translocation, intestinal microbiota, the intestinal barrier and cytokines in mesenteric lymph nodes and serum. Bacterial translocation decreased and gut dysbiosis improved in the probiotic group compared to the water group. The ileal β-defensin-1 concentration was higher and ileal malondialdehyde levels were lower in the probiotic group than in water group. There were no differences between groups in serum cytokines but TNF-α levels in mesenteric lymph nodes were lower in the probiotic group than in the water group. Fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 decreases bacterial translocation, gut dysbiosis and ileal oxidative damage and increases ileal β-defensin-1 expression in rats treated with CCl_4, suggesting an improvement in the intestinal barrier integrity.

Bacterial translocation of enteric bacteria to mesenteric lymph nodes or other extra intestinal sites can cause severe infections[1–3]. However, the interplay between these bacteria and/or their products and the host immune system can also contribute to the development of a proinflammatory state[1–3]. Inflammation is in turn related to multi-organ failure in critically ill patients[4], to metabolic diseases and their consequences[5], and to disease progression and development of complications in patients with liver diseases[1–3].

The mechanisms proposed to explain pathological bacterial translocation include alterations in gut microbiota, impaired intestinal barrier, and altered immune defenses[1–3]. Possible methods to prevent this phenomenon have been largely studied in experimental models of liver disease in rodents[6]. The administration of carbon tetrachloride (CCl_4) is one of the most widely used experimental models of liver disease and cirrhosis[7–10]. Several

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studies using this experimental model have demonstrated the high efficacy of antibiotics to decrease intestinal bacterial overgrowth and prevent bacterial translocation[11–13]. However, antibiotics favour the development of bacterial resistance, mainly if they are administered for long periods of time[14]. Therefore, alternative treatments have been proposed, including beta-blockers[15], bile acids[16], prokinetics[17], antioxidants[18] and probiotics[10,13,18].

Probiotics are living organisms that produce a beneficial effect to the host when administered in a sufficient amount[19]. They can prevent bacterial translocation by decreasing intestinal bacterial overgrowth and improving intestinal barrier and immune disturbances[20,21]. However, previous data on the prevention of bacterial translocation with probiotics in rodents treated with CCl_4 are contradictory. Although *Lactobacillus* GG[13] and *Lactobacillus johnsonii* La1[22] failed to show a benefit in bacterial translocation, other studies observed a reduction in bacterial translocation and proinflammatory state after treatment with *Bifidobacterium pseudocatenulatum* CECT7765 in Balb/c mice[10] and a multispecies probiotic combination in Sprague-Dawley rats[9]. These favourable results seem to be a consequence of an improvement in the intestinal barrier[9,10].

Lactobacillus paracasei subsp. *paracasei* CNCM I-1518 has been shown to improve the gut barrier and to reduce pro-inflammatory cytokines in Peyer's patches and bacterial translocation in experimental colitis[23,24]. However, this probiotic has not yet been evaluated in experimental cirrhosis. Actimel® (Danone, Palaiseau, Cedex, France) is a commercial dairy product widely used in humans that contains fermented milk with *L. paracasei* subsp. *paracasei* CNCM I-1518, in addition to the yogurt bacteria *Streptococcus thermophilus* and *L. bulgaricus*, and vitamins B_6 and D.

The aim of the present study was to compare the effects of fermented milk containing *L. paracasei* subsp. *paracasei* CNCM I-1518 (Actimel®) with water on bacterial translocation, gut microbiota, intestinal barrier and inflammatory response in the experimental model of rats treated with CCl_4.

Results

Fifty-four rats were included in the study. Forty-four rats were treated with CCl_4 and 5 died before week 6. At week 6 of treatment with CCl_4, the remaining thirty-nine rats were randomized into the probiotic group ($n = 20$) or the water group ($n = 19$). The remaining 10 rats made up the control group.

The time elapsed between randomization and the study end (death or laparotomy, performed 1 week after rats developed ascites) was similar in the probiotic group and in the water group (5.1 ± 0.6 vs 5.8 ± 0.8 weeks, $P = 0.60$). When considering only the rats that reached laparotomy, the time elapsed was 6.3 ± 0.7 and 6.1 ± 0.8 weeks, respectively ($P = 1$).

During the study, the total dose of CCl_4 received per rat was similar in the probiotic group and in the water group: 2873.0 ± 357.2 vs $2986.3 \pm 447.4\,\mu L$ ($P = 0.94$).

The mean daily volume of water with probiotic treatment that the rats in the probiotic group drank was 57.8 ± 3.3 mL/day. The mean daily volume of water that the rats in the water group drank was 27.1 ± 1.3 mL/day ($P < 0.001$ with respect to probiotic group). The mean daily volume of water that the rats in the control group drank was 34.6 ± 0.8 mL/day ($P < 0.001$ with respect to the probiotic group and $P = 0.001$ with respect to the water group).

The weight of chow that was eaten by the rats during the study was 21.3 ± 0.9 g/day in the probiotic group, 25.9 ± 1.2 g/day in the water group ($P = 0.01$ with respect to probiotic group) and 29.5 ± 1.3 g/day in the control group ($P < 0.001$ with respect to probiotic group, $P = 0.08$ with respect to water group). Therefore, the kcals received with the chow were lower in the probiotic group (61.8 ± 2.8 kcal/day) than in the water group (75.1 ± 3.6, $P = 0.01$) and the control group (85.5 ± 3.9, $P < 0.001$, $P = 0.08$ with respect to water group). As the rats in the probiotic group received a supplementary 20.5 ± 1.2 kcal/day with the fermented milk diluted in water, the total kcals received by the rats did not differ between the three groups (82.4 ± 3.3 in the probiotic group, 75.1 ± 3.6 in the water group, and 85.5 ± 3.9 in the control group, *P* NS). The weight of the rats at laparotomy was also similar in the two groups treated with CCl_4 (353.3 ± 30.9 g in the probiotic group and 388.1 ± 11.5 g in the water group, $p = 0.39$).

The estimated mean daily dose of bacteria received by rats in the probiotic group was $2.9 \pm 0.1 \times 10^9$ colony forming units (cfu).

Probiotic treatment did not reduce mortality or ascites formation. The mortality rate during the study was 35% (7/20) in the probiotic group, 31% (6/19) in the water group ($P = 0.8$ with respect to probiotic group), and 0% (0/10) in the control group ($P = 0.06$ with respect to the other two groups). The number of rats that developed ascites demonstrated by paracentesis was 80% (16/20) in the probiotic group and 79% (15/19) in the water group ($P = 0.9$). The cumulative probability of developing ascites at week 18 was 88% in the probiotic group and 86% in the water group ($P = 0.52$).

Probiotic treatment reduced bacterial translocation. Figure 1 shows the incidence of bacterial translocation was significantly lower in the probiotic group (7.7%, 1/13) than in the water group (54%, 7/13) ($P = 0.03$). The incidence of bacterial translocation was higher in the water group than in control rats (0%, 0/10) ($P = 0.007$). Table 1 shows the bacteria and the sites where bacteria were isolated. *Escherichia coli* and *Enterococcus* spp. were the most frequently detected bacteria in cirrhotic rats, mainly in the mesenteric lymph nodes.

Effect of probiotic treatment on intestinal microbiota. Figure 2 shows the concentration of enterobacteria and enterococci in the ileal and cecal contents in the three study groups analysed by microbial cultures. The two groups of rats treated with CCl_4 showed higher bacterial counts than control rats. This difference was statistically significant for ileal ($P = 0.03$) and cecal enterobacteria ($P = 0.01$) in the probiotic group, and for ileal enterobacteria ($P = 0.002$) and enterococci ($P = 0.03$) and cecal enterobacteria ($P = 0.02$) in the water group.

Group	Rat	Isolated bacteria	Laparotomy samples				
			Mesenteric lymph nodes	Ascitic fluid	Pleural fluid	Liver	Spleen
Water group	1	*E. coli*	+	−	−	−	+
	2	*E. coli* *Enterococcus* spp	−	−	−	+	−
	3	*E. coli* *Enterococcus* spp.	+	−	+	−	−
	4	*E. coli*	+	−	−	−	−
	5	*E. coli*	+	−	−	−	+
	6	*E. coli* *Enterococcus* spp.	+	+	−	−	−
	7	*E. coli*	+	−	−	−	−
Probiotic group	1	*E. coli* *Enterococcus* spp.	+	−	−	−	+

Table 1. Bacteria isolated in microbiological culture from samples of homogenizate of mesenteric lymph nodes, ascitic fluid, pleural fluid, liver and spleen in cirrhotic rats treated with water or probiotic. +represents presence of bacteria in microbiological culture, and − represents absence of bacteria in microbiological culture. *E. coli*, *Escherichia coli*.

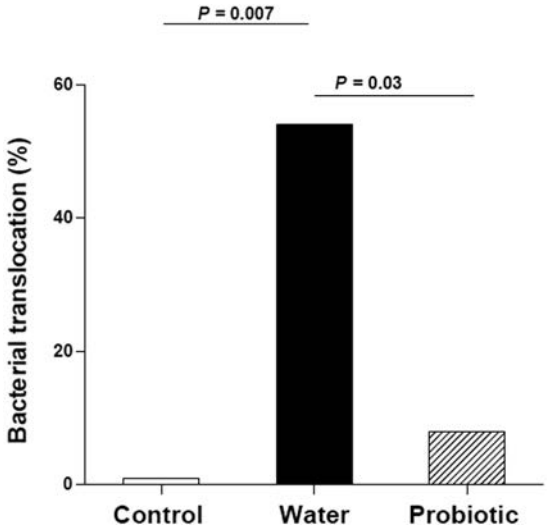

Figure 1. Incidence of bacterial translocation. Control rats (control group) ($n = 10$), rats treated with CCl_4 and water (water group) ($n = 19$) and rats treated with CCl_4 and fermented milk containing *Lactobacillus paracasei* ssp *paracasei* CNCM I-1518 (probiotic group) ($n = 20$). CCl_4, carbon tetrachloride.

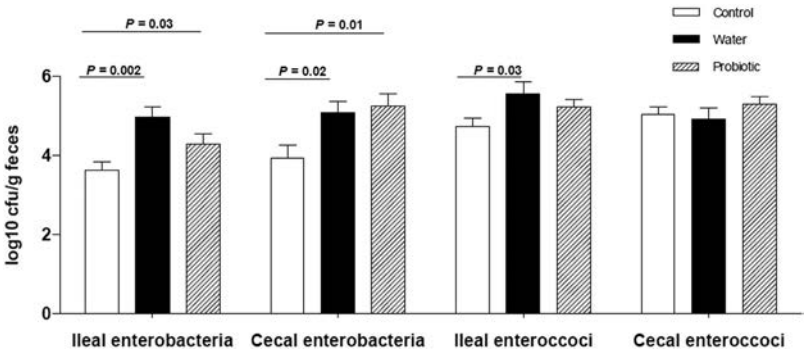

Figure 2. Bacterial concentration in ileal and cecal content. Control rats (control group) ($n = 10$), rats treated with CCl_4 and water (water group) ($n = 19$) and rats treated with CCl_4 and fermented milk containing *Lactobacillus paracasei* ssp. *paracasei* CNCM I-1518 (probiotic group) ($n = 20$). Values are mean ± SEM. cfu, colony forming units. CCl_4, carbon tetrachloride.

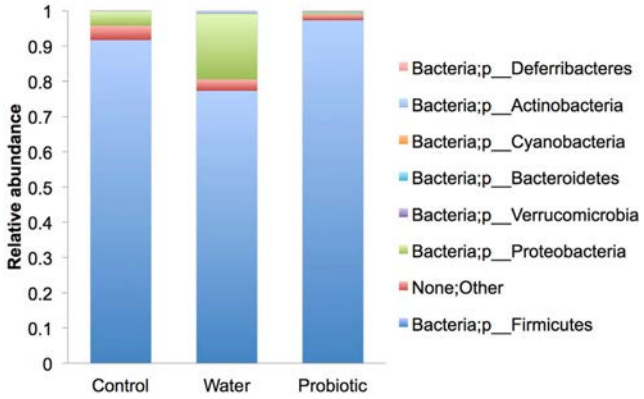

Figure 3. Relative abundance of the microbiome community at the phylum level. Firmicutes was shown to be significantly different between the water group compared to the control and the probiotic groups ($P = 0.01$; FDR = 0.07).

However, we did not observe differences between the probiotic group and the water group in any of the microbiological determinations.

However, the microbiome evaluation of ileal content by 16S rRNA analysis showed at the phylum level that the relative abundance of Firmicutes was significantly lower in the water group than in the control and probiotic groups ($P = 0.01$; FDR = 0.07) (Fig. 3). Moreover, there was a non-significant trend to a higher abundance of Proteobacteria in the control and the probiotic groups than in the water group.

Probiotic treatment reduced tumor necrosis factor-alpha (TNF-α) in mesenteric lymph nodes but did not change serum cytokines. Figure 4a shows cytokine concentrations in mesenteric lymph nodes. Interleukin-6 (IL-6) ($P = 0.01$) and TNF-α ($P = 0.001$) were higher and interleukin-10 (IL-10) ($P = 0.03$) was lower in the water group than in the control group, and TNF-α was lower in the probiotic group than in the water group ($P = 0.02$). Figure 4b shows ascitic fluid cytokine concentrations. There was a trend for a lower TNF-α ($P = 0.09$) and statistically significant lower IL-10 levels ($P = 0.04$) in the probiotic group than in the water group. Figure 4c shows serum cytokine concentrations. TNF-α levels were higher in the two groups treated with CCl_4 than in the control group ($P = 0.01$ with respect to the probiotic group and to the water group). No differences in serum cytokine concentrations were found between the water and the probiotic groups.

Probiotic treatment did not modify body weight and spleen/rat weight ratio. Body weight at laparotomy was higher in the control group (484.2 ± 20.6 g) than in the two groups of CCl_4 treated rats ($P < 0.001$), but it was similar in the probiotic group (353.3 ± 30.9 g) and in the water group (388.1 ± 11.5 g) ($P = 0.39$). The spleen/body weight ratio was lower in control rats (0.0026 ± 0.0001) than in the two groups of rats with cirrhosis ($P = 0.001$), and it was similar in the probiotic group (0.0059 ± 0.0006) and in the water group (0.0066 ± 0.0003) ($P = 0.45$).

Probiotic treatment increased β-defensin-1 and decreased malondialdehyde (MDA) in ileal samples. Figure 5 shows the expression of ileal occludin, claudin-4, zonula occludens-1 and β-defensin-1 were lower in the water group than in control rats. Ileal MDA levels were higher in the water group than in control group. Rats receiving probiotic treatment showed an increase in ileal β-defensin-1 and a decrease in ileal MDA with respect to the water group ($P = 0.04$ and $P = 0.01$, respectively). There were no statistical differences between probiotic and water groups regarding ileal occludin, claudin-4 and zonula occludens-1. Considering all the rats treated with CCl_4, ileal β-defensin-1 expression was lower in rats with bacterial translocation than in rats without (ratio 0.017 ± 0.004 vs 0.064 ± 0.018, $P = 0.04$). Moreover, we found a negative correlation between ileal β-defensin-1 and MDA ($r = -0.59$, $P = 0.006$).

Probiotic treatment did not modify liver damage. Figure 6 shows the degree of liver damage assessed by the histological score, Sirius red staining and the degree of steatosis. The two groups of CCl_4 treated rats had higher histological scores and a higher percentage of Sirius red staining than control rats. However, we did not find differences in these parameters between the probiotic group and the water group. We did not observe statistical differences between the three groups in the degree of steatosis.

Discussion

The main finding in this study was that probiotic treatment with fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 decreased bacterial translocation in rats treated with CCl_4.

Previous experimental studies evaluating probiotics in rodents treated with CCl_4 reported contradictory results. Two studies failed to show an effect of *Lactobacillus* GG[13] or *Lactobacillus johnsonii* La1[22] on bacterial translocation. However, Moratalla *et al.*[10] observed a decrease in bacterial DNA translocation in animals treated with *Bifidobacterium pseudocatenulatum* CECT7765 in an experimental model of Balb/c mice with CCl_4-induced

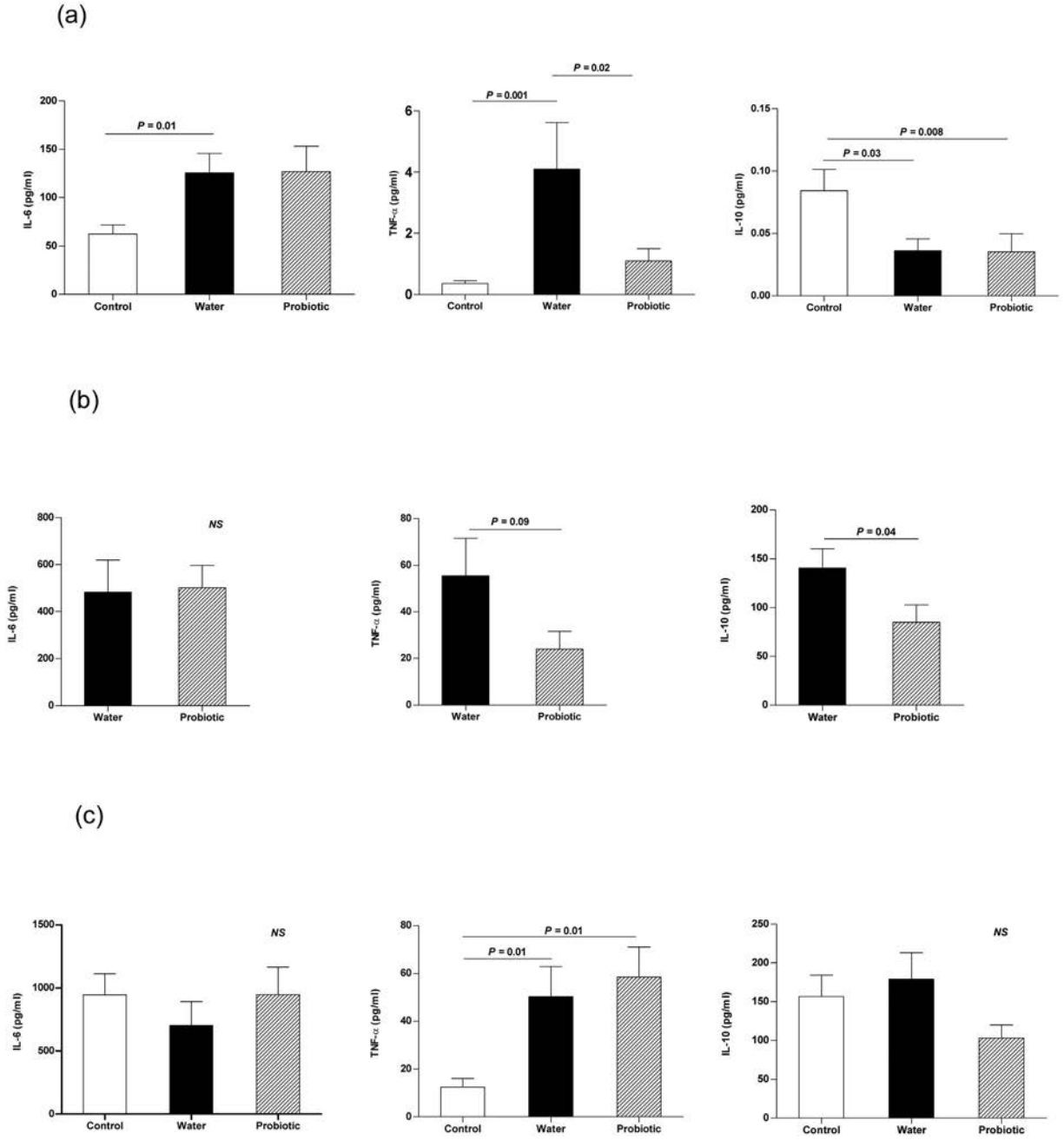

Figure 4. Inflammatory response. Control rats (control group) ($n = 10$), rats treated with CCl_4 and water (water group) ($n = 19$) and rats treated with CCl_4 and fermented milk containing *Lactobacillus paracasei* ssp. *paracasei* CNCM I-1518 (probiotic group) ($n = 20$). (**a**) Mesenteric lymph nodes, (**b**) ascitic fluid, and (**c**) serum. Values are mean ± SEM. CCl_4, carbon tetrachloride, IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-10, interleukin-10.

cirrhosis submitted to an oral overload of *E. coli*. Moreover, our group recently reported a decrease in bacterial translocation in CCl_4-treated rats receiving a multispecies probiotic mixture[9].

These contradictory findings could be due to several factors. First, different probiotics may produce different effects in a given experimental or clinical situation[16–18]. Second, differences among studies in the experimental model and in the duration of probiotic treatment[9,10,13,22] could explain some contradictory results. For the present study, we chose *L. paracasei* subsp. *paracasei* CNCM I-1518 because treatment with this probiotic has previously shown to reduce bacterial translocation in rats with colitis induced by the instillation of trinitrobenzene sulphonic acid[24]. With respect to the treatment schedule, we started probiotic administration at week 6 of CCl_4 administration and the rats received the probiotic until one week after development of ascites, for a mean of 6 weeks. We used this schedule because it is similar to that used in our previous study that showed a decrease in bacterial translocation with a different probiotic treatment[9].

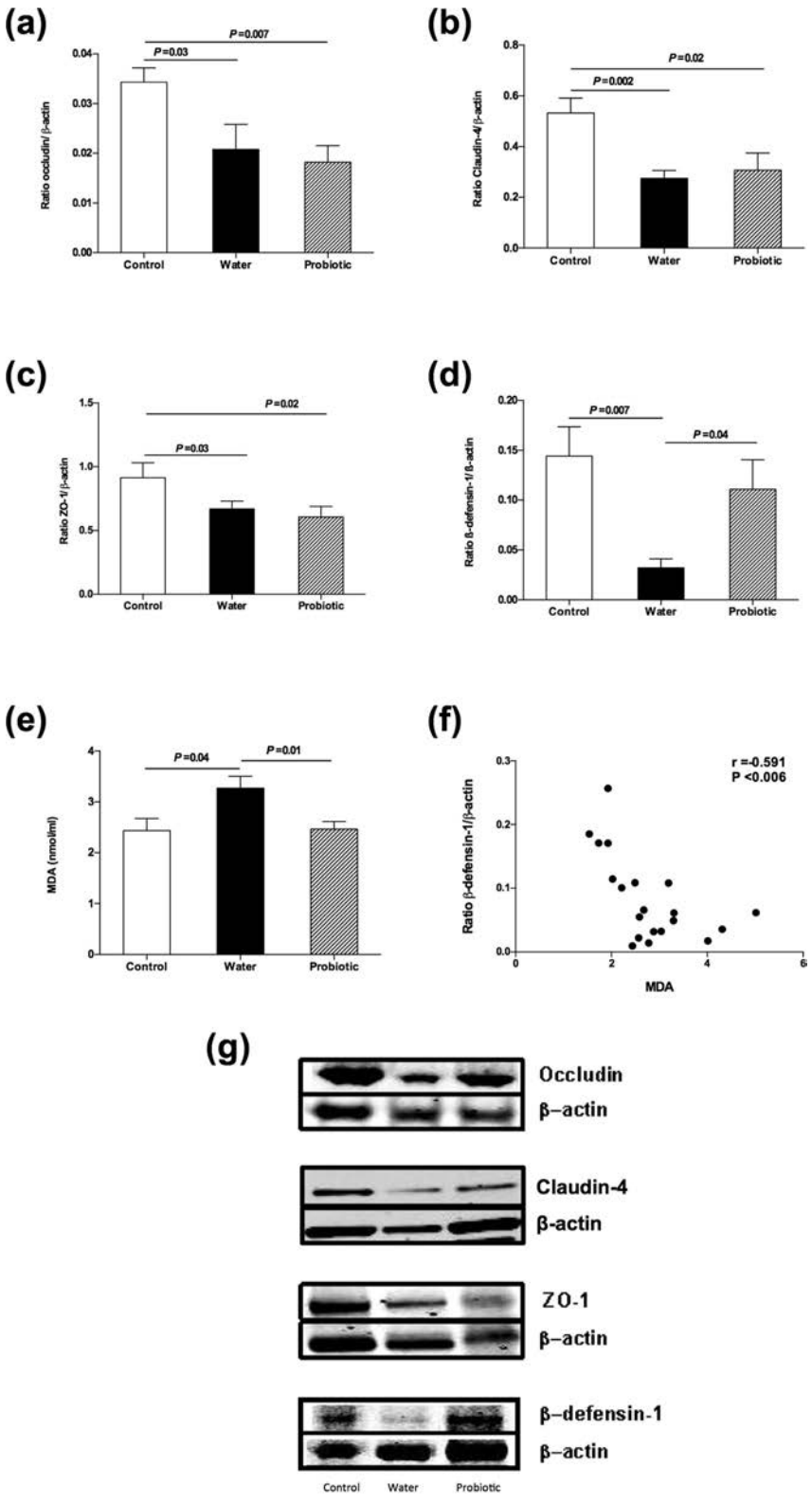

Figure 5. Intestinal barrier. Control rats (control group) ($n = 10$), rats treated with CCl_4 and water (water group) ($n = 19$) and rats treated with CCl_4 and fermented milk containing *Lactobacillus paracasei* ssp. *paracasei* CNCM I-1518 (probiotic group) ($n = 20$). Western blot of ileal samples for (**a**) occludin, (**b**) claudin-4, (**c**) zonula occludens (ZO)-1 and (**d**) β-defensin-1, respectively, (**e**) ileal MDA levels, (**f**) correlation between ileal expression of β-defensin-1 and MDA, and (**g**) representative image of western blot of occludin, claudin-4, ZO-1 and β-defensin-1 in control, water or probiotic group. Values are mean ± SEM. CCl_4, carbon tetrachloride, MDA, malondialdehyde. Cropped membranes are displayed and full-length blots are included in Supplementary Information.

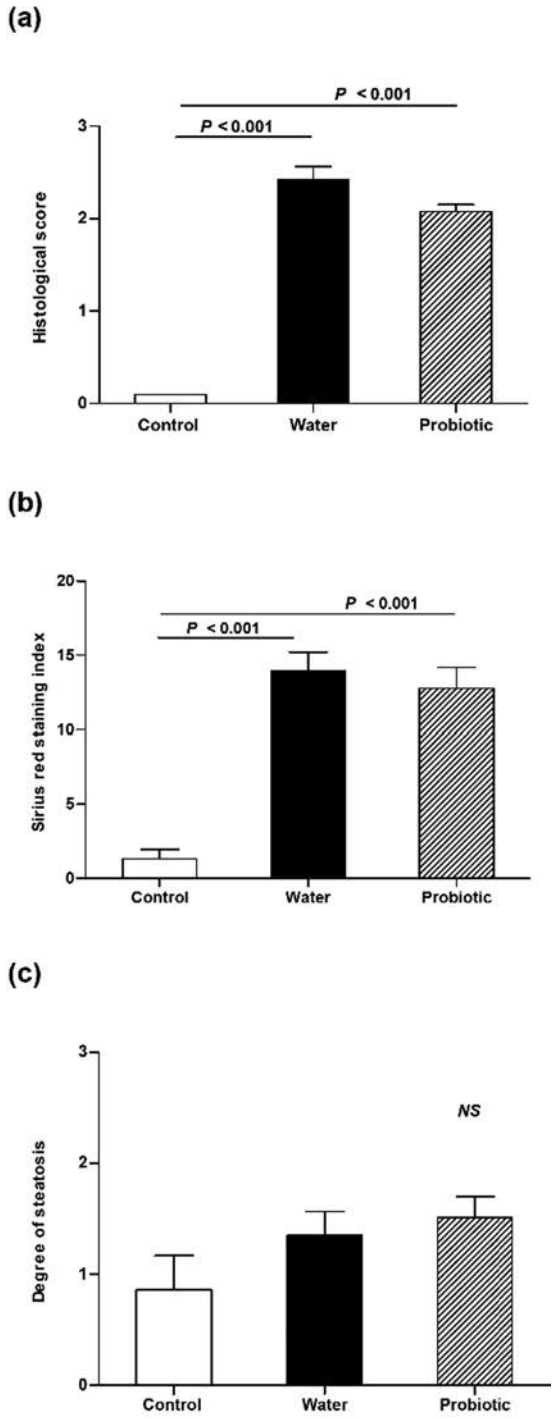

Figure 6. Liver damage. Control rats (control group) ($n = 10$), rats treated with CCl_4 and water (water group) ($n = 19$) and rats treated with CCl_4 and fermented milk containing *Lactobacillus paracasei* ssp. *paracasei* CNCM I-1518 (probiotic group) ($n = 20$). (**a**) Histological score, (**b**) Sirius red staining, and (**c**) degree of steatosis. Values are mean ± SEM. CCl_4, carbon tetrachloride.

The decrease in bacterial translocation observed in the probiotic group in the present study could be due to several non-excluding mechanisms: a decrease in intestinal bacterial overgrowth, an improvement in the intestinal barrier, or modulation of immune response[1–3]. Using microbiological culture methods, we found the intestinal concentration of the bacteria most frequently translocated in our study (enterobacteria and enterococci) was similar in the probiotic group and in the water group. However, the 16S rRNA analysis of the ileal content showed a significant increase in the relative abundance of Firmicutes and a non-significant trend to a decrease in Proteobacteria in the probiotic group in comparison with the water group. As cirrhotic rats from the water group presented a decrease in Firmicutes and a trend to an increase in Proteobacteria with respect to control healthy

rats, our findings suggest that the reduction in bacterial translocation that we observed in the probiotic group is partially due to an improvement in the gut dysbiosis.

Dysfunction of the intestinal barrier is considered a relevant mechanism to explain pathological bacterial translocation[1–3]. Tight junctions and antimicrobial peptides are important components of the gut barrier, and alterations in these components may contribute to bacterial translocation[1,25,26]. Few data, however, are available in cirrhosis[1,27,28]. In CCl_4-induced cirrhotic rats, Teltschik *et al.*[26] observed a relationship between bacterial translocation and deficiencies in the intestinal expression of Paneth cell α-defensins, mainly cryptidin 5 and 7, together with elevated or unchanged non-Paneth cell β-defensins expression. In the present study, we observed a decrease in the ileal expression of tight junction proteins, such as claudin-4, occludin and zonula occludens-1, and the antimicrobial peptide β-defensin-1 in the water group in comparison with control rats. Similar findings have been previously described by our group[9]. Probiotic treatment restored the decreased β-defensin-1 expression without significant changes in tight junction proteins. Other authors have also reported an association between the decrease in bacterial translocation and the increase in the expression of intestinal antimicrobial peptides, including β-defensin-1, in another experimental model, in mice submitted to repeated restraint stress undergoing moderate exercise[29]. In an experimental model of dextran sulphate sodium-induced colitis in Balb/c mice, *L. paracasei* subsp. *paracasei* CNCM I-1518 has been observed to improve the intestinal barrier, the gut permeability and the expression of zonula occludens-1[23].

Moreover, in the present study we found a decrease in the oxidative damage in the ileum of rats from the probiotic group. Intestinal oxidative damage has been associated with impairment in the intestinal barrier, delayed intestinal transit time[30,31], and bacterial translocation in rats treated with CCl_4[32]. Our findings suggest that the increase in β-defensin-1 expression and the decrease in oxidative damage in the gut barrier are relevant for explaining the reduction in bacterial translocation observed in the group treated with the probiotic.

Interestingly, in a previous study using the same experimental model, rats treated with a different probiotic mixture showed a decrease in bacterial translocation and an increase in occludin ileal expression, without changes in β-defensin-1[9]. These data suggest that different probiotics could have the same effect (in this case, a decrease in bacterial translocation) via different mechanisms.

Pathological bacterial translocation has been related to the proinflammatory state. In our study, rats treated with the probiotic presented a decrease in the levels of TNF-α in mesenteric lymph nodes. Other authors have also observed in an experimental model of dextran sulphate sodium-induced colitis in mice, that *L. paracasei* subsp. *paracasei* CNCM I-1518 reduced the production of pro-inflammatory cytokines in Peyer's patches[23]. This immune modulation could be due to the interaction between probiotic bacteria and the immune system[33,34] in mesenteric lymph nodes and/or to the decrease of bacterial translocation to the mesenteric lymph nodes by potentially pathogenic bacteria, such as enterobacteria and enterococci[1–3]. However, these changes in the mesenteric lymph nodes in the probiotic group were not associated with differences in the serum cytokine profile. Probably as a consequence of this lack of systemic effect, we did not observe significant variations either in the spleen/body weight ratio as a surrogate marker of portal pressure and hemodynamic disturbances, or in the incidence of ascites in the probiotic group with respect to the water group. In a previous study using the same experimental model, we observed that treatment with a different multispecies probiotic was accompanied by a decrease in serum TNF-α, spleen/body weight ratio and ascites formation[9].

Bacterial translocation and a proinflammatory state are relevant for promoting liver damage[1,3,35]. In our study, however, in spite of the reduction in bacterial translocation observed in the probiotic group, we did not find any significant effect on liver damage as assessed by the histological score and collagen deposition. This finding can be related to the absence of an effect of the probiotic treatment on the systemic inflammatory profile.

This study has a major limitation. We wanted to evaluate a commercial dairy product that contained not only *L. paracasei* subsp. *paracasei* CNCM I-1518 but also the yogurt bacteria *S. thermophilus* and *L. bulgaricus*, components resulting from milk fermentation, vitamins B_6 and D, and the milk itself. We can not therefore be sure if the effects observed in the probiotic group are due to *L. paracasei* subsp. *paracasei* CNCM I-1518 or to any other components of the mixture. It has been suggested that supplementation with vitamin D can protect against bacterial infections due to its immunomodulatory effects[36]. However, when evaluating the effects of a fermented product it is difficult to appraise the relative contribution of the different components, some of which are generated during the fermentation process[37]. Moreover, we are aware that we induced a significant nutritional change in the rats in the probiotic group. These rats received double the oral volume of fluid than the rats from the water group received, and although the mean total daily kcals were similar, the composition of the diet in the probiotic group was clearly different from that in the water group. This could affect the results, as the impact of the diet on the gut microbiota and bacterial translocation is well known[5]. Finally, to avoid the risks and interference with results that daily instrumentation could produce in such a relatively long-term study, we did not administer the probiotic by gavage but ad libitum in drinking water. This could make it difficult to know the exact dose of probiotic received by the rats. However, we recorded the total daily volume of the probiotic treatment that the rats drank and the calculated mean daily amount of bacteria administered was in the range of the scheduled dose.

In conclusion, our findings show that fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 decreases bacterial translocation, gut dysbiosis and ileal oxidative damage and increases ileal β-defensin-1 expression in rats treated with CCl_4.

Methods

Animals. We included Male Sprague-Dawley rats (Harlan Laboratories). They were individually caged and exposed to a 12:12 light/dark cycle and were on a constant room temperature of 21 °C. They were allowed free access to rat chow (A04, SAFE, Augy, France) and drinking water. This study was approved by the Animal Research Committee at the Institut de Recerca of Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) and by the Departament d'Agricultura, Ganaderia, Pesca, Alimentació i Medi Natural (DAAM) de la Generalitat de

Catalunya. Animals received human care and all methods were performed in accordance with the relevant guidelines and regulations according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals[38].

CCl_4 administration. We administered CCl_4 following Runyon *et al*'s method[39] to induce cirrhosis. Rats weighing 100–120 g were fed standard rodent chow (A04, SAFE, Augy, France) and treated with 1.5 mM/L phenobarbital (Luminal, Kern Pharma S.L., Barcelona, Spain) in drinking water to potentiate the effect of CCl_4. When they reached a weight of 200 g, weekly doses of CCl_4 (Sigma-Aldrich Química S.L., Tres Cantos, Madrid, Spain) were administered into the stomach using a stainless steel feeding tube (Popper and Sons, New Hyde Park, NY, USA) and a sterile pyrogen-free syringe (KD Medical GMBH Hospital, Berlin, Germany), without anesthesia. The initial dose of CCl_4 was 20 μL and subsequent doses were calculated according to the weight changes at 48 hours after the last dose, as previously reported[8].

Experimental design. At 6 weeks, the rats under induction of cirrhosis by the administration of CCl_4 were randomized to receive milk fermented by *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 in drinking water (probiotic group) or drinking water alone (water group) until laparotomy. Another group of healthy rats without induction of cirrhosis receiving drinking water (control group) was also included. All the rats from the three groups were allowed free access to rat chow and water with or without the probiotic (probiotic group, and water and control groups, respectively). A group receiving non-fermented milk was not included in the present study because we aimed to compare probiotic administration with the standard care, and milk is not included in the standard care of the rats in this experimental model. Mortality and the development of ascites during the study were recorded. In rats receiving CCl_4, laparotomy was performed one week after the development of ascites confirmed by paracentesis. The moment to perform the laparotomy in control rats was decided by matching with rats receiving CCl_4.

Administration of *L. paracasei* subsp. *paracasei* CNCM I-1518. The fermented milk (Actimel®, Danone, Palaiseau, Cedex, France) contained the probiotic strain *Lactobacillus paracasei* subsp. paracasei CNCM I-1518 combined with two bacteria commonly used as yogurt starters, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The microbial concentration of the fermented milk at the end of shelf-life met the target of 1×10^8 cfu/g of *L. paracasei* subsp. *paracasei* CNCM I-1518. *S. thermophilus*, and *L. bulgaricus* were also present in the final product at levels $>10^7$ cfu/g. The product used in this study also contained 0.42 mg of Vitamin B6 and 0.75 μg of vitamin D per 100 mL.

The product was diluted 1:1 with drinking water to achieve a final bacterial concentration of 0.5×10^8 cfu/mL. The doses were prepared in 100 mL containers every 12 hours during all the study. This schedule was chosen taking into account the mean daily volume of water that rats usually drink, in order to achieve the administration of a daily dose of probiotic of $1–5 \times 10^9$ cfu, previously used with other probiotics in experimental studies[9,13,18]. Rats drank ad libitum and the total volume of mixture they drank every 12 hours was recorded to check the dose of probiotic administered to rats. We also recorded daily the volume of probiotic mixture or water that the rats drank, the weight of chow they ate, the total kcals received, and the proportion of kcals corresponding to chow and to fermented milk.

Laparotomy. On the last day of the study, we performed a laparotomy under anesthesia with 10 mg/kg of xylacine (Rompun, Bayer, Leverkusen, Germany) and 50 mg/kg of ketamine (Ketolar, Parke-Dawis, New York, NY, USA) under sterile conditions. Samples of ascitic fluid, mesenteric lymph nodes, blood, liver, spleen, ileal stools and terminal ileal wall, cecal stools, and pleural fluid were collected in this sequence. Rats were euthanized using intravenous sodium thiopentate (Penthotal, Abbott Laboratories, Chicago, IL, USA). Blood samples were collected in BD Vacutainer tubes (BDbiosciences, San Jose, CA, USA) containing EDTA and centrifuged. The supernatants were recollected and frozen at −80 °C for later analysis. The other samples were stored at −80 °C. The spleen/body weight ratio was calculated and used as surrogate marker of portal hypertension[32,40].

Bacterial cultures. Samples of homogenized mesenteric lymph nodes, ascitic fluid, pleural fluid, spleen and liver were inoculated on Columbia blood agar, Columbia CNA agar, and the chromogenic media CPS ID3 (BioMérieux, Marcy l'Étoile, France) and incubated for 48 h at 37 °C in an aerobic atmosphere. Isolates were presumptively identified according to their growth and morphology. Bacterial translocation was considered as the presence of a positive culture in mesenteric lymph nodes, liver, spleen, or ascitic or pleural fluids[18].

During laparotomy, samples of cecal and ileal content weighing 0.2 g were collected, homogenized and diluted with 2 mL of normal saline in sterile conditions. The gut bacterial concentration was quantified by performing serial decimal dilutions. Samples of 100 μL of each dilution were inoculated on Columbia blood agar, Columbia CNA agar, and the chromogenic media CPS ID3 (BioMérieux, Marcy l'Étoile, France). After incubation for 48 h at 37 °C in an aerobic atmosphere, the colonies were counted. Counts are expressed as $\log_{10}$ cfu/g of fresh fecal sample with a detection limit at 10^3 cfu/g[18].

Microbiome analysis. We analyzed the microbiome of ileal content from 33 rats (23 cirrhotic rats, n = 13 treated with the probiotic and n = 10 treated with water; and 10 control rats). All the samples were subjected to genomic DNA extraction using a previously described method[41]. To chemically lyse the samples, we added 250 μl of 4 M guanidine thiocyanate, 40 μl of 10% N-lauroylsarcosine and 500 μl of 5% N-lauroylsarcosine before incubation at 70 °C. Further mechanical disruption was carried out using a Mini-Beadbeater-16 (Biospec Products©) to extract the DNA. To clear lysates, enzymatic digestion of RNA was performed by additing of 2 μl of a 10-mg/ml solution of RNAase, and the resulting DNA was precipitated and further ethanol-purified. Pure DNA was re-suspended in 200 μl Tris-EDTA buffer.

To analyse bacterial composition, we subjected extracted genomic DNA to PCR-amplification of the V4 hypervariable region of the 16S rRNA gene as previously described[41]. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain) according to the manufacturer's instructions, and further quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber©). The purified amplicons were pooled in equal concentration and finally subjected to sequencing using the Illumina Miseq platform at the Autonomous University of Barcelona (UAB, Spain).

For microbiome analysis, we loaded the raw sequences into the QIIME 1.9.1 pipeline and performed the quality filtering analysis as previously described[42]. After filtering, from 33 fecal samples, we obtained a total of 496761 of high-quality sequences with a number of reads ranging from 1435 to 40735 per sample. We used the USEARCH[43] algorithm to cluster similar filtered sequences into Operational Taxonomic Units (OTUs) based on a 97% similarity threshold. We then identified and removed chimeric sequences using UCHIME[44]. Since each OTU can comprise many related sequences, we picked a representative sequence from each one. Representative sequences were aligned using PyNAST against Greengenes template alignment (gg_13_8 release), and a taxonomical assignment step was performed using the basic local alignment search tool (BLAST) to map each representative sequence against a combined database encompassing the Greengenes and PATRIC databases. To correctly define species richness for the analysis of between-sample diversity, known as beta diversity, the OTU table was rarefied at 4849 sequences per sample.

Serum and mesenteric lymph nodes cytokine levels. Serum supernatants and extracts from mesenteric lymph nodes were tested for IL-6, TNF-α and IL-10 concentrations using specific ELISAs (Peprotech, London, UK), according to the instructions of the manufacturer. The detection limit was 30 pg/mL.

Intestinal barrier. To perform western blot analysis, protein was extracted from rat ileum using RIPA buffer following the protocol of the manufacturer (Sigma Aldrich, St. Louis, MO, USA). For this study, 20 μg of proteins were separated on a 4–12% SDS-PAGE (Invitrogen, Camarillo, CA, USA) gel and transferred to nitrocellulose. Membranes were incubated overnight with antibodies to claudin-4 (Invitrogen), occludin, zonula occludens-1 and β-defensin-1 (Antibodies-online Inc., Atlanta, GA, USA). We used appropriate secondary antibodies conjugated to IR-dyes 800CW goat anti-rabbit immunoglobulin G (IgG) and 680LT goat anti-mouse IgG (H + L) (Li-cor, Lincoln, Nebraska, USA) to visualize proteins. Proteins were then scanned using the Odyssey Imaging System (Li-cor). The expression of claudin-4, occludin, zonula occludens-1 and β-defensin-1 was quantified and normalized to β-actin using β-actin antibody (Sigma Aldrich). Oxidative damage in ileal samples was assessed using the determination of MDA formation by thiobarbituric reaction[32,45]. The detection limit of this assay was 0.079 nmol/mg protein.

Liver damage. Four μm-slices from paraffin blocks were evaluated by hematoxylin-eosin staining to study the histological changes and by Masson's trichrome staining to assess the severity of fibrosis. A semi-quantitative score was used by a single expert pathologist to blindly classify the liver samples into: 0- normal, 1- fibrosis with thin and incomplete fibrous tracts, 2- regeneration nodules with thin complete fibrous tracts, and 3- regeneration nodules and thick and complete fibrous tracts. The degree of steatosis was evaluated in: 0- normal, 1- mild steatosis, and 2- severe steatosis. The degree of hepatic fibrosis was estimated by calculating the percentage of the area stained with picro-Sirius Red (Sirius Red F3B, Gurr-BDH Lab Supplies, Poole, England). The positive stained area was quantified using a morphometric analysis system. Briefly, twelve images were obtained with an optic microscope (Nikon Eclipse E600, Nikon Corporation, Japan) at magnification of x20. Images were imported using an image-analysis system (AnalySIS, Soft-Imaging System, Munster, Germany) software and automatically merged. The positive area was the sum of the area of all positive pixels.

Statistical analysis. Statistical analysis was performed using the SPSS statistical package (SPSS Inc. version 17.0, Chicago, Illinois, USA). All parameters are reported as percentages and mean $\pm$ SEM. Differences between groups were analyzed using the Fisher's exact test for qualitative variables. The Shapiro-Wilk test was used to check the normality of data distribution. The Student's t-test was used when data were normally distributed; otherwise, the non-parametric Mann-Whitney U-test was used. Correlations were calculated using Pearson's test or Spearman's test. The probability of developing ascites was assessed by Kaplan-Meier test and then compared using the log rank test. A two-tailed *P* value below 0.05 was considered statistically significant.

Specifically regarding the microbiome evaluation, statistical analyses were carried out in QIIME and in R. To work with normalized data, we analyzed an equal number of sequences from all groups. The Kruskal-Wallis one-way test of variance was used to compare the mean number of sequences of the groups. The analysis provided false discovery rate (FDR)-corrected *P*-values. FDR < 0.1 was considered significant for all tests.

Sample size was calculated according to previous data from our group[15]. Considering an α error of 0.05, a β error of 0.20, an expected percentage of bacterial translocation of 62% in the water group and of 8% in the probiotic group, and a 43% overall mortality during the study, we calculated that the minimal number of rats required in each group was 19.

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Author Contributions

E.S., S.V., H.C., C.G. and G.S. designed research; E.S., J.C.N., F.J.S., P.S.B., and B.M. conducted research; P.S.B., B.M. and C.J. provided essential reagents; A.S., X.M., and C.M. performed microbiome analysis, E.S., J.C.N., S.V. and G.S. analyzed data and performed statistical analysis; E.S., J.C.N. and G.S. wrote the manuscript; S.V., C.J. and C.G. critically reviewed the manuscript; G.S. had primary responsibility for final content. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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