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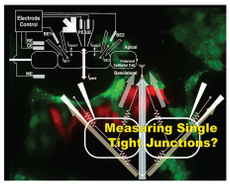

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REVIEW

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Role of microbiota and related metabolites in gastrointestinal tract barrier function in NAFLD

Maria Victoria Fernandez-Cantos[a*], Diego Garcia-Morena[a*], Valeria Iannone[b*], Hani El-Nezami[c], Marjukka Kolehmainen[b**], and Oscar P. Kuipers[a**]

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ABSTRACT

The Gastrointestinal (GI) tract is composed of four main barriers: microbiological, chemical, physical and immunological. These barriers play important roles in maintaining GI tract homeostasis. In the crosstalk between these barriers, microbiota and related metabolites have been shown to influence GI tract barrier integrity, and alterations of the gut microbiome might lead to an increase in intestinal permeability. As a consequence, translocation of bacteria and their products into the circulatory system increases, reaching proximal and distal tissues, such as the liver. One of the most prevalent chronic liver diseases, Nonalcoholic Fatty Liver Disease (NAFLD), has been associated with an altered gut microbiota and barrier integrity. However, the causal link between them has not been fully elucidated yet. In this review, we aim to highlight relevant bacterial taxa and their related metabolites affecting the GI tract barriers in the context of NAFLD, discussing their implications in gut homeostasis and in disease.

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NAFLD; GI tract microbiota; microbial metabolites; GI tract barriers; intestinal permeability

1. Introduction

The GI tract is responsible for the processing of food and allows the survival of commensal microorganisms while eliminating pathogens.[1] The GI tract is a complex structure composed of four main barriers: microbiological, chemical, physical and immunological, which all play key roles in the maintenance of the system homeostasis. The gut microbiota modulates the pool of metabolites present along the GI tract.[2] Depending on their origin these metabolites can be classified into three types: (1) metabolites resulting from the transformation by the microbiota of dietary components (e.g. Short-Chain Fatty Acids, SCFAs);[3] (2) host secreted metabolites which are modified by the microbiota (e.g. secondary bile acids)[4] and (3) metabolites synthesized *de novo* (e.g. vitamin K).[5] All these metabolites can affect different processes in the GI tract of the host.[2] For instance, butyrate,[6] tryptophan derivatives[7] or ethanol[8] have been shown to affect the intestinal permeability with different mechanisms. Perturbations in GI barrier functions might lead to increased gut permeability; a condition also termed "leaky gut", which, in turn, would allow for a higher flow of bacteria and bacterial products to the bloodstream, contributing to liver damage.[9–11] Non-alcoholic fatty liver disease (NAFLD) is an umbrella term encompassing several liver disorders. In individuals who consume little or no alcohol, nonalcoholic fatty liver (NAFL) can progress from simple liver steatosis to nonalcoholic steatohepatitis (NASH). Typically, NASH is accompanied by pericellular fibrosis, which may further progress to irreversible cirrhosis and ultimately hepatocellular carcinoma.[12–14] Its prevalence has increased worldwide and doubled over the last 20 years, as it is thought to be present in more than 20% of the general population, particularly affecting people suffering from Type 2 Diabetes Mellitus (T2DM).[15] Although the mechanisms behind the progression of NAFLD are not well understood yet, several factors contribute to its development and

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progression. Alterations in the GI microbiota (e.g. changes in microbiota composition, species diversity, community structure and/or function), age, diet and lifestyle, host metabolism, and genetic predisposition are thought to play an important role. These risk factors have been addressed in several reviews.[13,16,17]

The important role of the gut microbiota in NAFLD has been established both in preclinical and clinical studies.[10,18] Qualitative and quantitative alterations in the microbiota impact the GI barrier function and structure, increasing GI tract permeability, contributing to liver damage. In fact, patients with NAFLD show increased intestinal permeability that correlates with the severity of steatosis.[11,19,20] Moreover, recent research has shown that the disruption of the GI vascular barrier is also required for NAFLD pathogenesis.[19] Most of the NAFLD studies are focused on establishing a correlation between bacterial microbiota composition and the disease state, but the causal mechanisms linking them have not been fully elucidated. For that purpose, in the present review, we aim to highlight relevant bacterial metabolites and taxa affecting the GI tract barriers in NAFLD. Their implications in gut homeostasis and in disease will be discussed. Consequently, this review can contribute to understanding NAFLD pathogenesis, further elucidating the mechanisms that lead to gut barrier dysfunction associated with NAFLD.

2. GI tract structure and function

The GI tract is composed of different segments reflecting their specific physiological functions.[21] In this review, we will focus specifically on small and large intestines. The small intestine includes duodenum, jejunum and ileum. Its main functions are the digestion and absorption of nutrients from the diet.[22] The large intestine, the last part of the GI tract, is formed by cecum, proximal colon, distal colon and rectum and its primary functions are absorbing water, electrolytes and forming luminal content for elimination.[23] The large intestine is also the place where commensal bacteria produce vitamins and Short Chain Fatty Acids (SCFAs).[1] The GI tract plays important roles in maintaining the homeostasis between the internal and external environments and it is composed of several barriers: 1) the microbiological barrier composed of the commensal microorganisms;[24] 2) the chemical barrier represented by secreted molecules that provide a defense against invading microorganisms;[25–27] 3) the physical barrier regulating nutrient uptake and protecting the intestinal mucosa from pathogen invasion;[28–30] and 4) the immunological barrier including a variety of immune cells, which, during pathogenic conditions, are mobilized to fight and clear invading microbes (Figure 1).[31] This review focuses on the structure and function of the microbiological, chemical and, with more emphasis, physical barriers. The role of the immune system in the GI tract as a barrier will be not described in this review. There are several excellent and extensive reviews on the role of the mucosal immune system, which we recommend for further reading, but are outside the scope of this review.[31–33]

2.1. Microbiological GI tract barrier

The first and outer GI tract barrier is represented by intestinal microbiota, referring to the entire population of microorganisms colonizing the GI tract (Figure 1).[24] It includes not just bacteria, but also fungi, archaea, yeasts and viruses, that have a mutualistic relationship among themselves and with their host.[34] The symbiotic relationship between microbiota and host has several effects on the GI tract physiology: 1) it influences the mucosal innate and adaptive immune system;[35] 2) it provides metabolic functions assisting the digestion and fermentation of food that is important for the production of vitamins and SCFAs;[5] and 3) it also provides defense against pathogens competing for food and adhesion sites in the GI tract, some even actively eliminating competitors by secreting antimicrobial peptides (AMPs).[36] Qualitative or quantitative alterations in the microbiota can impair this symbiotic relationship, decreasing the GI tract barrier protection.[37,38] These alterations have been shown to play a role in NAFLD and will be discussed more in-depth later in this review. It is noteworthy that here terms like “gut microbiota” refer to the totality of the organisms inhabiting our GI tract, leaving a potential higher-than-expected role in gut-related disorders to eukaryotic microorganisms, protozoa, archaea and parasites, all of which have been highly understudied in the past years.[39,40] Recent research in the virome highlighted

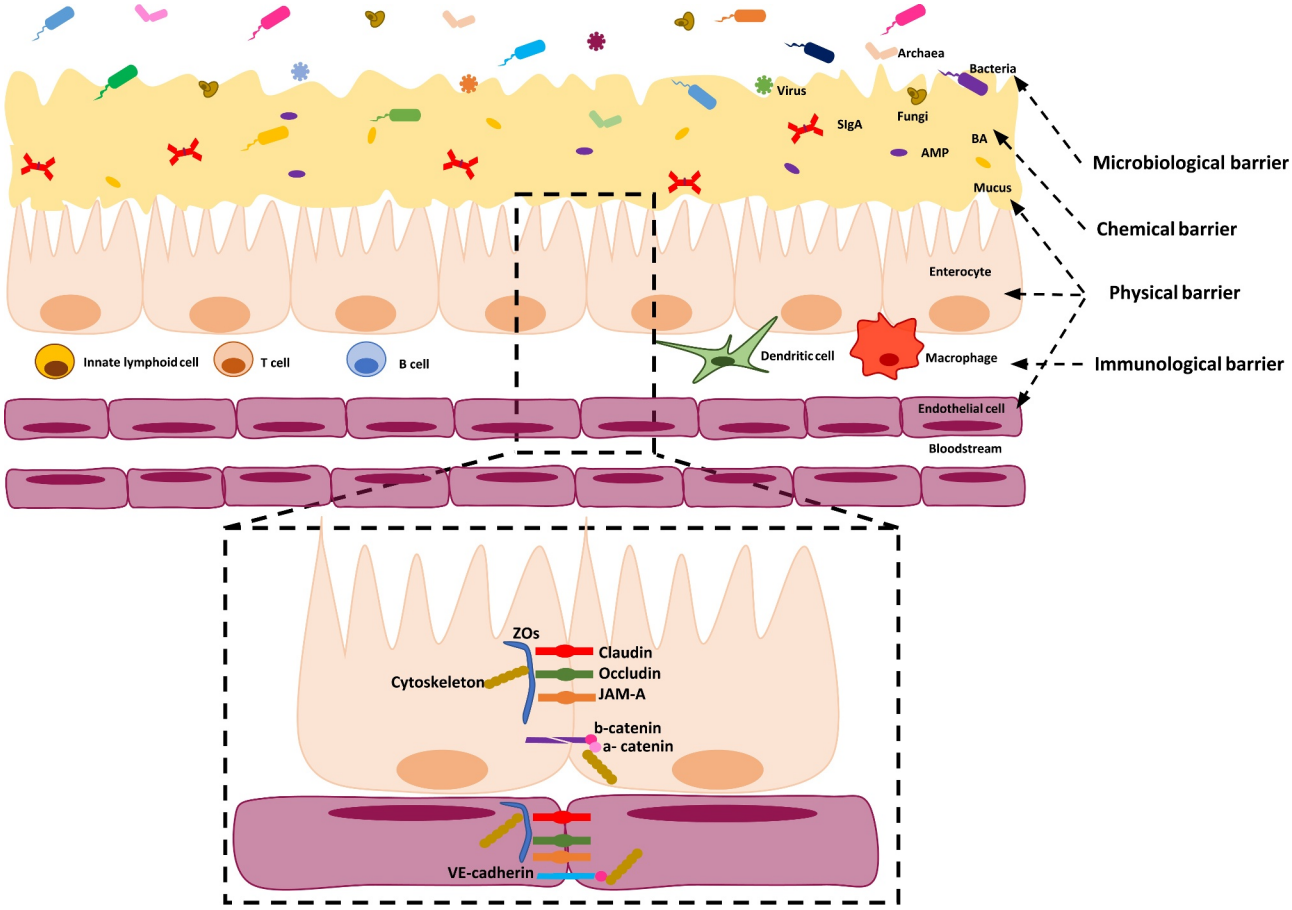

Figure 1. Structure of microbiological, chemical, physical and immunological barriers in the intestine. The microbiological barrier resides in the intestinal lumen, over and inside the mucus. It is composed of intestinal microbiota including bacteria, fungi, archaea and viruses. The mucus layer forms part of the physical barrier (coating with a single layer the small intestine and with a double layer the large intestine), in which AMP, BA and SIgA are secreted and they act as chemical barriers. The mucus covers the intestinal epithelial cells, which are also considered part of the physical barrier. Moreover, these cells are tightly sealed by TJs (claudins, occludin, ZOs), JAM-A and by AJs (E-cadherin) controlling the paracellular permeability. Scattered throughout the epithelium and lamina propria, innate immune cells (i.e. macrophages, dendritic cells and lymphoid cells) and adaptive immune cells (i.e. T and B cells) reside. In the lamina propria of the gut mucosa, endothelial cells tightly sealed by claudin, occludin, JAM-A and VE-cadherin proteins form a gut vascular barrier.; AJs: adherens junctions, AMP: antimicrobial peptides, BA: bile acids, SIgA: Immunoglobulin A, JAM-A: TJs-associated adhesion molecules, TJs: tight junctions, VE-cadherin: vascular-endothelial cadherin, ZOs: zonula occludens.

that not only bacterial diversity is reduced in NAFLD, but that the indicators of NAFLD also correlate with significant decreases in viral diversity and in the proportion of bacteriophages.[40] On the other hand, You *et al.*,[39] recently reviewed the current knowledge of intestinal fungi in different liver diseases. They reported that an imbalance in fungal populations also occurs in patients with metabolic syndrome and that oral supplementation with *Saccharomyces boulardii* alleviated hepatic steatosis and inflammation. The under-studied role of viruses and fungi has affected the way we use terms like "gut microbiota" or "microbiome", which can easily be misused, or misinterpreted to represent only a portion of the total biodiversity of the GI-tract. For example, the microbiome is more than the bacteriome.[41] A more precise term should then be used when speaking about the changes in (only) bacterial composition or abundance. Hence, in this review, we will use the term bacterial gut microbiota (BGM) to stress the difference from general statements about the gut microbiota as a whole.

2.2. Chemical GI tract barrier

The chemical barrier in the GI tract is composed of secreted molecules, such as host secreted AMPs,[25] bile acids (BAs)[26] and secretory Immunoglobulin A (SIgA)[27] (Figure 1). BAs are derivatives of

cholesterol metabolism and they are considered to have a direct antimicrobial effect by destroying bacterial membranes because of their detergent-like properties.[42] The function of BAs will be further explained later in this review. AMPs are mainly produced by enterocytes and Paneth cells, and also macrophages placed in the gut mucosa can contribute to their production.[43–45] The number of Paneth cells changes along the GI tract, with the highest amounts in the ileum.[46,47] Paneth cells are lacking in the large intestine, which leads to a different AMPs composition in the large intestine compared to the small intestine.[48] The most common secreted AMPs in the GI tract include defensins (α and β), cathelicidins (LL-37/CRAMP) and C-type lectins (RegIII α/γ/β). AMPs target components of the bacterial cell wall, although different AMP families use different mechanisms to exert their antimicrobial activity, including enzymatic and non-enzymatic activities.[25] AMPs expression is regulated by diverse mechanisms and some of them require bacterial signals for their expression. For instance, bacteria and bacterial products, but not fungi or protozoa, induce the release of some AMPs, such as α[49] and β defensins.[50] AMPs also control the access/interaction of the gut microbiota with the intestinal mucosa as shown, for example, in mice lacking RegIIIγ, in which altered mucus distribution has been observed, as well as increased contact of microbiota with intestinal epithelium and inflammation in ileum mucosa.[51] Dysfunctions in AMPs activity have also been observed in intestinal bowel diseases. Additionally, AMPs have been shown to play an important role in host-microbiota interaction[52] and in promoting barrier integrity.

Inside the mucus, SIgAs are the main immunoglobulins secreted on the intestinal mucosal surface and contribute to maintaining the homeostasis in the intestine. SIgAs are produced by plasma cells inside the mucosa, transported by the polymeric Ig receptor (pIgR) inside IECs and secreted into the lumen. SIgAs are able to neutralize invading microorganisms mainly interfering with the microbial adherence to IECs by forming intraluminal immune complexes. This process, defined as immune exclusion, prevents colonization and damage to IECs. SIgAs can also neutralize the invading pathogens that penetrate inside the lamina propria of the mucosa.[53]In addition, SIgAs are able to modulate the microbiota composition participating in the maintenance of GI tract homeostasis.[54] And, their importance in GI tract has been shown in activation-induced cytidine deaminase (AID) deficient mice in which the SIgAs production is lacking due to the missing switch from Immunoglobulin M (IgM) to IgA. In the intestine of these mice an hyperplasia of lymphoid follicles has been observed suggesting that SIgAs are essential in preventing hyper-stimulation of the mucosal immune system.[55] Despite the importance of SIgA in the GI tract, individuals with IgA deficiency do not present specific symptoms. This could be explained as a compensatory mechanism by an increased production of IgM observed in the intestine of these individuals.[56] But it has been also reported that individuals with IgA deficiency could suffer from pulmonary infections, allergies and GI tract disorders.[57]

2.3. Physical GI tract barrier

The physical barrier in the GI tract consists of mucus layers, IECs and endothelial cells (ECs) (Figure 1). They physically avoid the invasion of microorganisms through the mucosa, preventing the translocation of bacteria and bacterial components to the bloodstream.[28–30] However, their features vary between the small and large intestine. The small intestine has a smaller radius than the large intestine, and its epithelium is folded forming villi (evaginations projected toward the intestinal lumen) and crypts (invaginations occurring at the base of the villi). Intestinal stem cells found at the base of the crypts divide, migrate and differentiate continuously into enterocytes, goblet cells, Paneth cells or enteroendocrine cells, whose physiological roles are respectively the absorption, mucus production, antimicrobial production and hormone secretion. Besides, crypts contain mainly stem cells and Paneth cells, while enterocytes are predominantly present in the villi, and enteroendocrine and goblet cells are distributed in the upper crypt and the villi. In contrast, the large intestine lacks Paneth cells and villi, and only crypts are present along it.[58] Moreover, the interaction of the different cell types with the microbiota will not be equal, as well as, the concentration of the molecules secreted by the host will differ along the radius of the intestine.

2.3.1. The mucus layer barrier

Mucus represents the first physical barrier of the GI tract mucosa and its distribution varies between the small and large intestine. In the small intestine, a single and easy to remove layer of mucus fills up the space between the villi and covers them. However, in the small intestine, the mucus is not the main and essential mechanism of defense due to the presence of high amounts of AMPs close to the epithelium.[28] In fact, the mucus in the small intestine allows bacteria to attach to it as well as to obtain nutrients from it, while it also acts as a viscous medium for the chemical barrier (AMPs and SIgA).[59] In contrast, the large intestine is covered by two mucus layers, an inner and an outer layer. The inner layer is dense, directly in contact with the epithelium and only a specific crypt-associated microbiota is able to penetrate the mucus and thrive next to the epithelium,[60] while the outer mucus layer has similar properties to the mucus layer found in the small intestine. The outer layer, which derives from the inner layer, forms a less dense net-like structure where most of the microbiota reside.[28] Irrespective of its location in the small or large intestine, the mucus is mainly composed by the glycoprotein mucin 2 (MUC2) forming a viscous net. Goblet cells are the producers of MUC2. These cells are able also to produce other minor mucins in the intestine (i.e. MUC1, MUC3, MUC17), the intestinal trefoil factor (ITF), the AMP resistin-like molecule β (RELMβ), and Fc-γ binding protein (Fcgbp).[59] The importance of the mucus as a barrier has been shown in murine models, in which the lack of MUC2 leads to direct contact of microbiota with the intestinal mucosa and to severe colitis.[61] Interestingly, Bergstrom *et al.*[62] have recently proposed a novel spatial and dynamic organization of mucus in different segments of the large intestine. The proximal colon has been shown as the main producer of MUC2 and responsible for lumen content (microbiota and feces) encapsulation, while the distal colon produces a locally minor amount of MUC2 which adheres with that one received from the proximal colon. Moreover, the microbiota in the proximal colon is able to control the MUC2 production,[62] suggesting a new dynamic and continuous mucus system in the colon.

2.3.2. Intestinal epithelial cells barrier

The second physical barrier is composed of IECs, which are organized in a monolayer of columnar epithelial cells. The permeability of this barrier is regulated by both paracellular and transepithelial permeability. Paracellular permeability refers to the passage of molecules through the space between the cells, allowing the passage of small molecules, ions and water, and being impermeable to most other entities.[29] Transepithelial permeability regulates the transport of solutes across the IEC.[8]

The paracellular permeability is mainly regulated by Tight Junctions (TJ) and the TJs-associated junctional adhesion molecules (JAM), residing in the apical surface of IECs (Figure 1). Relevant members of TJs are occludin, claudins, zonula occludens (ZOs). All of them, with the exclusion of ZOs, are membrane proteins and they extend into the paracellular space between epithelial cells.[63] Occludin was the first member identified of the TJ complex.[64] Its function is not yet fully understood, but both *in vivo* and *in vitro* studies indicate that it might have a role in promoting the integrity of the epithelial barrier in the intestine.[65] Claudins, a large family of transmembrane proteins, are responsible for the regulation of paracellular permeability between epithelial cells, mainly promoting intestinal barrier integrity.[66,67] While some of the family members are associated with increased paracellular permeability to sodium and water.[68,69] A good balance among the various isoforms is needed for maintaining the barrier integrity, as they have differential expression along the GI tract.[70] JAMs are glycoproteins of the immunoglobulin superfamily composed of two extracellular immunoglobulin-like domains, one transmembrane domain and one cytoplasmic.[71] JAMs are expressed in different cell types, including immune cells, and colocalize with TJs in epithelial and endothelial cells.[72] For this reason, they are often wrongly called TJs, although they are TJ-associated proteins. The JAM molecules A and −4 are involved in the maintenance of the barrier integrity. ZOs[73,74] are intracellular scaffold proteins and link TJs[75] and JAM-A[76] to the cytoskeleton. ZOs belong to the family of membrane-associated guanylate kinase (MAGUK)-like proteins and include three different isoforms (ZO-1, 2 and 3).[77]

The cytoskeleton contraction is regulated by myosin light chain (MLC) and its phosphorylation by kinases such as Myosin light chain kinase (MLCK) results in the opening of the paracellular pathways.[78] Therefore, aberrant expression levels of this kinase can reduce the TJs barrier function, since MLCK-mediated MLC phosphorylation leads to TJ loss. Several aspects regarding their function and role in health and pathological conditions remain to be investigated.

Zonulin is a protein that has been shown to be a key player regulating TJs and it is strongly associated with increased intestinal permeability.[75] Zonulin is released mainly as a consequence of the intestinal lumen exposure to bacteria[79] and to gliadin.[80] Gliadin is a gluten component which has been suggested to bind the chemokine receptor CXCR3 receptor on IECs. This leads to the recruitment of the adapter protein MyD88 and, subsequently, the expression of zonulin.[80] Zonulin is mostly present in the jejunum and distal ileum, but absent in the large intestine[77] and it is able to disassemble the TJs via polymerization of actin in a protein kinase C (PKC) dependent way.[78] Circulating levels of zonulin are considered an important intestinal permeability marker and has been linked to diseases, such as Type 1 Diabetes[79] and celiac disease.[80]

Besides TJs controlling the paracellular permeability, the junctions complex between epithelial cells is also characterized by adherens junctions (AJs) and desmosomes. Both of them have transmembrane domains (cadherins and desmosomal cadherins) supporting the adhesion between epithelial cells and in the cytoplasm respectively connected with the actin cytoskeleton through b and a catenin (AJs) and with intermediate filaments (desmosomes).[81] E-cadherin is the most characterized AJ and it plays an important role in maintaining intestinal homeostasis[82] and intestinal barrier integrity promoting the assembly of TJs.[83]

2.3.3. Intestinal endothelial cells barrier

ECs in the lamina propria form a third physical barrier. The gut vascular barrier (GVB) has been first described by Spadoni *et al.*[30] and it is composed of ECs surrounded by pericytes and enteric glial cells. The endothelial barrier shows similarities with the junctions complex observed in IECs. In fact, ECs are characterized by the presence of occludin, ZO-1, JAM-A and vascular endothelial cadherin (VE-cadherin), which control the GI vascular barrier integrity and the trafficking of solutes and fluids into the bloodstream.[30,84,85] PV1 is an integral membrane protein of ECs expressed when vascular permeability is increased[85,86] and modulated by Wnt/β-catenin pathway.[30] Spadoni *et al.*[30] also demonstrated that bacteria able to cross the IECs do not spread directly to the liver and spleen, due to this vascular GI barrier, thus providing evidence for the existence and importance of the GVB.[30] This physical barrier is not so profoundly studied as other physical barriers and its impact on diseases with intestinal mucosa disturbance followed by IECs increased permeability and/or disruption of the mucus layer/s deserves to be further investigated.

3. NAFLD and GI-tract microbiota

3.1. The gut microbiota changes during NAFLD

In NAFLD, it has been observed that several taxa of the BGM are either increased or decreased when comparing patients at different stages of the disease. This notion, as well as, further insights into NAFLD and fibrosis, their diagnostic tools and challenges; and a thorough analysis of the overlap of bacterial components of the gut microbiota between type 2 diabetes, obesity, fibrosis and NAFLD in human patients are further discussed in an outstanding, recent review.[87] In the following section, we will discuss these results with respect to BGM alterations and try to provide a link with damage or protection on the gut barrier. Of note, many of the physiological effects of the BGM could be attributed to their metabolites, which will be addressed later in this review.

In human studies (Supplementary Table 1), some concordant results have been reached when comparing patients with different liver conditions, as reported by Aron-Wisnewsky *et al.*[87] In particular, the γ-Proteobacteria phylum, the families *Pasteurellaceae* and *Enterobacteriaceae* and the *Escherichia* and *Shigella* genera belonging to the latter family, were increased both in NAFLD-fibrosis and in NAFLD. The same increase was observed for *Streptococcaceae, Veillonellaceae, Bacteroides* and *Ruminococcus*, but not for the

Ruminococcaceae family in general. In contrast, *Haemophilus* and *Eubacterium* appeared to be consistently decreased, as well as species of the above mentioned *Ruminococcaceae* family. Studies on patients with NAFLD-cirrhosis showed some concordant results, such as a decrease in *Clostridiales XIV, Lachnospiraceae, Eubacterium* and *Ruminococcaceae spp*; and an increase in *Escherichia* or *Veillonella*.[88–90] Of note is the increase in *Enterobacteriaceae*, consistently reported in different liver injuries. This increase contrasts with the low abundance of Proteobacteria sequences (including *Escherichia coli*) encountered in healthy individuals, where they may represent ~0.1% of the bacteria in the strict anaerobic environment of the colon.[91,92] An increase in species belonging to the *Streptococcus, Veillonella* and *Porphyromonas* genera, which typically comprise oral species, has also been reported before.[93,94] It seems that during the onset and/or progression of NAFLD, oral species are able to migrate and colonize the gut altering the community structure. Interestingly enough, a similar decreased relative abundance of *Lachnospiraceae* and *Ruminococcaceae*, paired with increased numbers of *Enterobacteriaceae, Streptococcaceae* and *Porphyromonadaceae*, are associated with hepatic encephalopathy, the major clinical manifestation of an altered gut-liver-brain axis.[95]

3.2. The impact of gut bacteria on intestinal permeability

3.2.1. The influence of lipopolysaccharide

The crosstalk between the GI tract and the liver has been widely addressed in the literature. In short, the GI tract-liver interaction is established by bidirectional communication via the biliary tract and the portal vein. BAs and other mediators are transported from the liver to the gut through the bile ducts, while bacterial metabolites, products and microbe-associated molecular patterns (MAMPs) translocate from the GI tract and reach the liver via the portal vein.[96] Therefore, intestinal permeability is a crucial aspect of maintaining homeostasis, and its perturbation could translate into different diseases. It has indeed been suggested that increased systemic inflammation could be associated with increased intestinal permeability, which would pose a higher risk factor for mortality and morbidity in older patients.[97] The inflammation-intestinal permeability interaction is likewise bidirectional: an increased intestinal permeability allows for a higher flow of MAMPs, such as lipopolysaccharide (LPS), pathogens and antigens leading to inflammation driven by the production of pro-inflammatory cytokines.[98] These cytokines can, in the gut epithelium, increment the intestinal permeability by altering TJs structure and paracellular permeability.[48,98,99] In fact, patients with NAFLD have a significantly increased intestinal permeability and alterations in the intestinal TJs in both the IECs and in the ECs, compared to healthy individuals.[11] The impairment in the integrity of the intestinal barrier and the GVB by the gut microbiome have been identified as prerequisites for the development of NASH.[19]

It is therefore not surprising that the association between BGM alterations, lower BGM diversity, NAFLD and increased MAMPs across the studies underlines the role of the BGM in the onset and development of metabolic liver diseases.[11,20,100,101] In particular, LPS has a critical role in triggering immune responses that lead to inflammation in different organs and to intestinal permeability, a phenomenon that usually happens during NASH and cirrhosis. Since LPS is one of the major components in the cell wall in gram-negative bacteria, the increased relative abundance of *Enterobacteria, Bacteroides*, or the invasion of oral gram-negatives could account for increased endotoxemia. However, recent research by d´Hennezel *et al.*,[102] shows how structural modifications of the LPS across members of the order *Bacteroidales*, the main LPS contributor along the gut, silences TLR4 signaling, facilitating host tolerance[102] and alters the proinflammatory paradigm of LPS.[103]

3.2.2. Intestinal microbiota and MUC2

Although secreted MUC2 is expressed constitutively by goblet cells, its production is upregulated by TLR-signaling to replenish the mucin that is degraded by commensals or removed by peristalsis.[104] Bacteria such as *Porphyromonas,*

Ruminococcus gnavus, Ruminococcus torques, Bacteroides or *Escherichia coli*, with higher relative abundances in NAFLD, as discussed before, can degrade MUC2 through different pathways,[105] enabling bacteria to colonize the epithelial surface. Therefore, TLR activation is vastly increased. This leads to an increase in the intracellular molecular pathways in IECs which end up in the activation of NF-κβ and the expression of inflammatory cytokines (TNF-α, IL-1β, IL-6, among others), chemokines, vasoactive factors (like nitric oxide) and reactive oxygen species (ROS).[106,107] These different cascades signal for the recruitment of immune cells that perpetuate inflammation.[107,108] Forming a vicious cycle of further gut barrier damage via TNF-α, which contributes to TJ dysfunction and malfunction in the synthesis of MUC2. Interestingly, some putative probiotic strains belonging to the *Lactobacillus* genus have been shown to bind to TLR4 and upregulate MUC2 expression in the HT-29 cell line model.[109] In fact, TNF-α, interleukin-4 (IL-4), and IL-13 can induce MUC2 transcription via NF-κβ.[110] This could indicate that a certain "basal" level of TLR excitation is required for the proper function of the gut barrier, and leaves open the question of how the gut epithelium is able to discriminate between maleficial and beneficial bacteria.

Intestinal microbiota can upregulate MUC2 transcription by providing the nutrients required for MUC2 syntheses, such as vitamins B, K, and D and amino acids.[105] Vitamin D is considered to have good predictive power for identifying NAFLD-cirrhosis, and its insufficiency has been associated with NAFLD.[111] Interestingly, a recent review[112] on long non-coding RNAs (lncRNAs) highlighted the importance of the expression levels of Vitamin D receptors (VDR). The authors speculated that increased levels of lncRNA H19, commonly occurring in various pathological conditions, could be related to the disruption of the epithelial barrier function by increasing the abundance of a microRNA (miR-675). miR-675 then targets VDR mRNA and decreases the levels of ZO-1 and E-cad mRNAs,[112,113] contributing to intestinal permeability.

Direct contact of pathogens with the IECs triggers the immune response and affects the TJ complex in several ways. In the following paragraphs, we will describe relationships between the alterations in the TJ protein function and NAFLD.

3.2.3. Alterations in function of zonula occludens, zonulin and TJs-associated adhesion molecules

In the case of ZO-1, it has been reported that adult patients with NAFLD present reduced ZO-1 expression in duodenum compared to healthy patients.[11] Moreover, increased small intestine bacterial overgrowth co-occurred in NAFLD patients with intestinal permeability, suggesting a link between intestinal permeability, bacterial overgrowth and hepatic steatosis.[11] Mouries *et al.*,[19] reported that ZO-1 expression was reduced as early as after 48 h following feeding mice with a high-fat diet (60%) inducing leakage of the gut, before the emergence of steatosis. Moreover, they reported that the maintenance of the gut leakage occurred throughout the development of NASH and inflammation-driven insulin resistance and obesity.[19] In another study, Pacifico *et al.*,[114] found that in children with NAFLD, zonulin levels also correlated with the severity of steatosis,[114] and zonulin levels correlate positively with pathological markers of NAFLD (such as body mass index, triglycerides and serum Il-6, among others).[115] Assimakopoulos *et al.*,[116] showed how the duodenal expression of occludin and claudin-1 were decreased in patients with cirrhosis, and even further decreased in patients with decompensated cirrhosis. Furthermore, the expression of both occludin and claudin-1 in patients with compensated or decompensated cirrhosis were inversely correlated with endotoxin concentration.[116] Unfortunately, none of the previous studies performed a BGM profiling, which precludes to identify BGM alterations that could be compared to those found in other NAFLD studies (section 3.1). It is of note that most of the studies of TJs alterations are performed in the duodenal region, while the microbiota profiling of the gut is based on fecal samples in the majority of the cases, which might hinder specific microbial alterations happening in the duodenal region.

Rahman and colleagues[117] reported reduced protein and transcript levels of JAM-A in the colon mucosa of NAFLD patients, which correlated with increased mucosal inflammation.[117] Analysis of the BGM content in JAM-A knockout mice (*F11r*$^{-/-}$) fed with high fructose and high cholesterol diet,

showed an increase in pro-inflammatory bacteria, mainly belonging to the Proteobacteria and Firmicutes phyla. Besides, they showed that depletion of microbiota protected $F11r^{-/-}$ mice from diet-induced NASH.[117] Since JAM-A controls the paracellular permeability and prevents LPS from crossing the epithelium, deficiencies in this protein made mice more susceptible to NASH in the presence of high proinflammatory pathobionts.

3.2.4. Alterations in E-cadherin function

As previously discussed, E-cadherin is a crucial AJ for intercellular associations, and its loss of function has been linked to several pathological processes. In fact, deletion of E-cadherin in mice resulted in severe weight reduction, loss of intestinal epithelial architecture and problems in the differentiation of secretory cell lineages.[118] In particular, Paneth cell maturation and placement along the intestine is affected by the loss of E-cadherin. The reduction in Paneth cell count impairs proper bacterial defense, facilitating the approach of pathobionts to the IECs. Interestingly, the enterotoxin fragilysin, produced by *Bacteroides fragilis*, can interact and cleave the extracellular domains of E-cadherin,[119] resulting in intestinal permeability. *Bacteroides* is a common resident in the gut and conflictive results are reported when assessing its contribution to liver disease (Supplementary Table 1). This bacterium, typically considered a commensal, has also been found to be responsible for a significant increase in the biofilm mass in patients with Crohn's disease.[120] However, the apparent pathological relation with Crohn's disease is as well challenged by Hsiao *et al.*[121] Treatment of the model maternal immune activation (MIA) mice with *B. fragilis* rendered an improvement in gut barrier integrity and promoted the restoration of the relative abundance of 6 operational taxonomic units in the absence of *B. fragilis* colonization. Remarkably, treatment with *B. fragilis* was able to correct autism spectrum disorder-related behavioral abnormalities in this mice model.[121] This highlights how members of the same genus or family contribute differently to the homeostasis of the intestine, blurring their role in health and in disease. How members of the same species account for different effects, possibly depending on the environmental cues (such as diet, or genetic background); as well as, how strain-level identification of the BGM is needed to account for strain-specific traits, are important outstanding questions.

3.2.5. Gut-vascular barrier disruption

Recently, Spadoni *et al.*[30] reported an increase of the GVB disruption in the small and large intestine of mice, after just one week fed with a high-fat diet, and in the colon of NASH patients. The disruption of the GVB, marked by PV1 upregulation, was parallel to increased permeability detected in murine IECs after just 48 hours of high-fat feeding. This suggests that GVB represents yet another barrier of defense that prevents bacteria or specific molecules from progressing to the systemic circulation. In fact, they proposed that disruption of the GVB (indicated by upregulated PV1) is a prerequisite for NASH pathogenesis and is a consequence of diet-induced microbiota changes.[19] Pathogens, such as *Salmonella* Typhimurium, interfere with the WNT/β-catenin pathway allowing them to bypass the GVB, suggesting that the WNT/β-catenin could be an important player in GVB barrier disruption.[19,30] More studies, focusing on the mechanism by which the GVB disruption happens and the role of the BGM are needed and represent an interesting approach for studying NAFLD pathogenesis.

4. The link between gut microbiota metabolites and gut barrier function in NAFLD

4.1. Ethanol

Due to the histological similarities between obesity-related fatty liver disease and alcohol-induced liver damage,[122] common pathogenic mechanisms have been suggested to be involved in both conditions. Consequently, it has been hypothesized that NAFLD patients may be exposed to endogenous ethanol, as has been shown in human patients[123,124] and mice[124] (Supplementary Table 2, Figure 2). For instance, pediatric NASH patients present higher serum levels of ethanol than obese and healthy children.[123]

GI tract microbiota has been suggested as the source of this endogenous ethanol, being able to produce it as a result of the fermentation of simple carbohydrates, including sucrose, glucose, and

fructose.[125–128] An excess of those carbohydrates in the diet and/or a decrease in gut motility (associated with obesity, diabetes, or chronic alcohol abuse) have been shown to enhance this pathway.[125] Intestinal stasis has been suggested to permit the overgrowth of colonic bacterial strains in the upper gastrointestinal tract, thus promoting the fermentation of those simple carbohydrates and yielding ethanol as a result.[129] Oral administration of the antibiotic neomycin in obese mice reduced breath ethanol excretion, suggesting the role of GI tract microbiota in the production of ethanol.[129] Moreover, Zhu *et al.*[123] associated the increase in blood ethanol levels with an increase in abundance of Proteobacteria/*Enterobacteriaceae/Escherichia sp*. Under anaerobic conditions and in the absence of alternative electron acceptors, *Escherichia coli* catalyzes the mixed-acid fermentation pathway converting sugars into several products, including ethanol.[130] In an interesting recent study, a high alcohol producing *Klebsiella pneumoniae* strain, but not strains unable to produce ethanol, was shown to induce NAFLD in mice. Its colonization was suggested to affect gut barrier function via decreasing expression of occludin and ZO-1.[131] The same study also reported an association between disease severity and high alcohol producing *Klebsiella pneumoniae* isolates in patients with NAFLD.[131] The results of this study point out the importance of studying the role of specific bacterial strains in the development of the disease, showing that the analysis to higher taxons such as phylum, order or genus are insufficient. On the other hand, other intestinal bacterial genera from different phyla have been shown to produce ethanol such as *Bacteroides*[137] and *Bifidobacterium*.[138] Nevertheless, the majority of researchers believe that fungi are the main contributors to the endogenous production of ethanol.[8]

Ethanol can be metabolized by both the host and the GI tract microbiota. On the one hand, ethanol is typically metabolized in the host through the so-called oxidative conversion. In this pathway, acetaldehyde is produced by the action of the enzyme alcohol dehydrogenase (ADH). Subsequently, the enzyme acetaldehyde dehydrogenase (ALDH) converts acetaldehyde into acetate.[8] This process takes place mainly in the hepatocytes, although these enzymes are also present in other tissues including the intestinal mucosa.[8] In addition, in the large intestine, the mucosal ALDH activity is lower than ADH activity and, hence, an accumulation of the toxic and reactive metabolite acetaldehyde is expected.[132] GI-tract microbiota has also been suggested to metabolize ethanol in an ADH-dependent manner, as it was shown for the *Enterobacteriaceae* family.[133] Other ubiquitous distributed host enzymes involved in ethanol metabolism to acetaldehyde are CYP2E1 and catalase.[134] In the intestine, nonoxidative alcohol metabolism via reactions with free fatty acids and membrane phospholipids has been described to occur. The former reaction generates fatty acid ethyl esters (FAEE) and the latter generates abnormal phospholipids affecting cell signaling of intestinal epithelial cells.[132]

The disruption of gut barrier function by ethanol has been mainly studied in the context of alcoholic fatty liver disease (AFLD) and it can do it through several pathways. First of all, ethanol consumption has been shown to alter GI tract microbiota composition[135,136] and function,[137] affecting the microbiological barrier. Regarding the functional alteration of the intestinal microbiota, a decrease in the SCFAs propionate and isobutyrate production has been described, as these metabolites are important for gut barrier integrity.[137] Furthermore, ethanol has been associated with the loss of gut-associated lymphoid tissues (GALT) cells in mice, compromising the clearance of potentially pathogenic bacteria such as *Salmonella typhimurium*.[138] Concerning the chemical barrier, the production of antimicrobial molecules by host cells has also been shown to be compromised by alcohol consumption. For instance, alcohol feeding has been associated with mice models with a downregulation of the antimicrobial C-type lectin RegIIIβ in the small intestine.[139]

However, the most relevant mechanism by which ethanol disrupts gut barrier function is associated with the physical barrier. It has been shown to disrupt the IECs themselves (transepithelial permeability) and the spaces between them (paracellular permeability).[8] Transepithelial permeability is mainly caused by: (1) cell death causing loss of epithelium mainly at the duodenum villi tips;[140] and (2) reactive oxygen/nitrogen species (ROS/RNS) production resulting from ethanol metabolism, which generates nitroxidative stress and cellular damage.[141] Paracellular permeability caused by alcohol involves its action in TJs. Cho

et al[141] showed a decrease in binge alcohol-exposed rat in the levels of TJs (ZO-1, claudin-1, −4, and occludin), AJs (β-catenin and E-cadherin) and desmosome plakoglobin. The same study found similar results for human subjects who suddenly died from heavy alcohol intoxication. In Caco-2 cells, an *in vitro* model of intestinal epithelial cell monolayers, alcohol is able to induce a change in expression of ZO-1, claudin-1 and JAM-A, thus increasing intestinal permeability.[142,143] Additionally, ethanol can affect gut cell structures via cytoskeleton modification.[144] Moreover, alcohol-related metabolites, such as acetaldehyde and FAEEs, have also been shown to cause TJs disruption. Acetaldehyde, the most toxic ethanol-associated metabolite, induces translocation of protein phosphatase 2A (PP2A) to TJs and dephosphorylates occludin threonine residues, preventing its interaction with ZOs, thus disassembling TJs.[145–147] This metabolite also inhibits tyrosine phosphatase activity, increasing tyrosine phosphorylation of ZO-1[148] and AJs (β-catenin and E-cadherin),[149] compromising cell-cell adhesion. FAAEs, such as ethyl oleate and ethyl palmitate, are able to increase permeability dose-dependently through disruption and decrease of ZO-1.[150]

Despite the histological similarities between both conditions, obesity-related fatty liver disease and alcohol-induced liver damage, the conditions and concentrations under which ethanol exerts its effect in the GI-tract could be potentially different. As a consequence, more studies about endogenous ethanol production under NAFLD conditions are needed in order to uncover its real influence in this pathology and in particular its potential contribution in GI tract barrier dysfunction.

4.2. Short-chain fatty acids (SCFAs)

SCFAs, which include organic acids with 1 to 6 carbons, are the main end products of colonic bacterial metabolism.[151] The enzymatic capability of humans is not able to degrade dietary fiber, leaving the complex carbohydrate present in there as substrates for anaerobic bacterial fermentation in the colon (Supplementary Table 2).[3,152] This fermentation process involves a variety of enzymatic reactions and metabolic processes, supporting colonic microbial growth and maintenance and yielding metabolic end products, such as SCFAs, that can be used by the host.[3] SCFAs produced include acetate, propionate and butyrate. Acetate is the most abundant SCFA, followed by propionate and butyrate.[3]

Some commensal bacteria have been identified to have an anti-inflammatory effect through the production of SCFAs. Among them, butyrate has been shown to play a key role in maintaining and enhancing gut barrier homeostasis,[153] being the entity most extensively studied. In general, this SCFA at a low concentration (2 mM) decreases the permeability to mannitol/inulin and increases transepithelial electrical resistance (TER) in Caco-2 cell monolayer.[154,155] However, its effect is considered as paradoxical, since at high concentrations (8 mM) it has the opposite effect, increasing permeability to inulin and reducing TER significantly.[154] The ability to produce butyrate has been mainly identified in bacteria classified into different families of the *Firmicutes* phylum, although some other producers are members from nine other phyla, including Actinobacteria, Bacteroidetes, Proteobacteria or Fusobacteria.[156]

Mechanistically, butyrate has been shown to promote gut barrier integrity affecting different GI tract barriers. At epithelium level, it directly interacts with IECs since it is preferentially used as intestinal fuel by colonocytes, preferred over other SCFAs, glucose or glutamine.[3,157] Metabolism of butyrate, and to a lesser extent acetate´s and propionate´s metabolism, depletes O_2 thus resulting in the stabilization of hypoxia-inducible factor (HIF).[158] HIF is a transcription factor that targets genes associated with antimicrobial defense,[159] mucin production,[160] and the ITF implicated in the repair of the intestinal mucosa.[161] In addition, butyrate can affect IECs enhancing their base crypt proliferation, an event associated with the prevention of the development of colon cancer.[162] However, not all the studies suggest a protective role of butyrate in colon cancer and this lack of agreement has led to the definition of the "butyrate paradox", suggesting that this discrepancy may be explained by differences in experimental design (excellently reviewed in Lupton, 2004).[163] In addition, butyrate promotes goblet cell differentiation by increasing the expression levels of Krüppel-like transcriptional factor 4 (KLF4).[164] This SCFA also stimulates the production of mucin via increasing MUC2 expression of goblet cells in *in vitro* models.[165,166] Paracellular permeability has also been

shown to be decreased by butyrate, accelerating TJs assembly through activation of AMP-activated protein kinase (AMPK) in Caco-2 cell monolayers.[167] The same study reported that 2 mmol/L butyrate did not significantly alter the expression of four different TJs (claudin-1, claudin-4, ZO-1 and occludin). Nonetheless, other studies have found an increase in the expression of occludin, ZO-1 and claudin-2.[168,169] With regard to the chemical barrier, butyrate also mediates AMPs production, binding specifically to the G-protein-coupled receptor 109A (GPR109A) in the colonic epithelium. As a consequence, IL-18 is produced,[170] a cytokine which has been shown to be involved in mucin production and AMPs production, such as intelectin-1 (ITLN1), RELMβ and members of the angiogenin family.[3,171] Nonetheless, IL-18 can control intestinal homeostasis or induce a pro-inflammatory response in a concentration-dependent manner.[172] Butyrate also binds to GPR43 in IECs and promotes the production of RegIIIγ and β-defensins.[173] Thus, butyrate promotes the expression of other AMPs such as human cathelicidin LL-37/human cationic antimicrobial protein 18 (hCAP18) in IECs[174,175] or calprotectin in intestinal macrophages.[45] In general, fermentation of SCFAs results in a decreased pH, which influences the microbiological barrier indirectly reducing, for instance, the abundance of potentially pathogenic clostridia.[3]

A clinical study carried out by Michail *et al.*[176] demonstrated that levels of acetate are decreased in fecal samples of obese children with NAFLD in comparison to healthy children and those obese without evidence of NAFLD. The same study did not report significant differences in butyrate and propionate levels. In contrast, Raman *et al*[177] detected an increase in all three types of SCFAs in fecal samples of obese humans with NAFLD. These latter findings are supported by another clinical study, where butyrate and propionate were enriched in fecal samples of patients with mild NAFLD, whereas acetate was enriched in adults with advanced fibrosis.[178]

Despite the association of SCFAs with the maintenance and enhancement of gut barrier function, these compounds have been shown to affect other tissues, such as adipose and hepatic tissues, and processes, including lipogenesis and gluconeogenesis (reviewed in Chu *et al.*[26]). As a consequence, depending on the mechanisms or signaling pathway that SCFAs activate, they appear to both prevent but also promote the development of NAFLD.

4.3. Bile acids

Bile acids (BAs) are soluble products derived from the catabolism of cholesterol. In mammals, there are two major classes of BAs: primary and secondary BAs.[179] Primary BAs, chenodeoxycholic acid (CDCA) and cholic acid (CA) are synthesized in the liver and then conjugated with glycine or, to a lesser extent, taurine.[42,179] Upon conjugation and after meal consumption, BAs are released into the duodenum.[42] Later in the ileum, BAs are reabsorbed and further re-circulated to the liver in the so-called enterohepatic circulation.[179] A small portion of BAs (~5%) escape this resorption and are deconjugated by the microbiota before either being absorbed or converted into secondary BAs.[180] The bacterial enzyme catabolizing this deconjugation is the bile salt hydrolase (BSH) and it is present in all major bacterial and archaeal divisions in the human GI tract, including members of the Firmicutes, Bacteroidetes and Actinobacteria phyla.[181] In the large intestine, GI tract microbiota further metabolizes deconjugated BAs through 7α-dehydroxylation, converting them into secondary BAs, which can be excreted or passively reabsorbed.[42,179] As a consequence, from CDCA lithocholic acid (LCA) is formed and from CA deoxycholic acid (DCA). *Clostridium* from clusters XIVa and XI and *Eubacterium* species have been described to be able to produce secondary bile acids.[42] In addition, *in silico* analyses, have shown that bacteria from clades *Ruminococcaceae, Lachnospiraceae* and *Peptreptococcaceae* exhibit bile acid-inducible 7α-dehydroxylation genes.[182] Some other isomerizations and oxidations can take place further diversifying the pool of secondary BAs.[42] Consequently, the presence or absence of bacterial species able to catalyze these transformations will be critical in determining the pool of BAs present in the host.

The BA pool composition is relevant since the hydrophobicity of BA species (UDCA<CA<CDCA<DCA<LCA) is an important determinant of their cytotoxic effect.[183] The greater the hydrophobicity, the greater the toxicity, probably via an indirect mechanism that involves oxidative stress induction and the production of ROS species.[183] Additionally,

BAs can modulate different receptors, each of which shows different affinities and effects depending on the BA species.[180] As a consequence, the composition of the pool of BAs is critical in the activation of different signal pathways and, hence, the GI tract microbiota is a critical factor in host physiology. However, not only the GI tract microbiota affects the pool of BAs, but also the latter can shape the bacterial community by inhibiting the growth of sensitive bacteria and promoting those able to metabolize BAs. BAs are considered part of the chemical GI tract barrier, having direct antimicrobial activity due to their detergent properties.[42,184] Gram-positive bacteria are generally more sensitive to the antimicrobial effect of BAs than Gram-negative bacteria, although tolerance to BAs has been shown to be a strain-specific feature.[185] Nevertheless, BAs also exert indirect effects modulating the activation of the farnesoid X receptor (FXR), which has been shown to induce the transcription of inducible NO synthase (iNOS), IL-18 and angiogenin (ANG1); all of them are involved in antibacterial defense.[186]

The occurrence of NAFLD has been associated in clinical studies with dysregulation of BA homeostasis (Supplementary Table 2, Figure 2). Some studies have described an increase of total BAs in serum levels in NASH patients.[187,188] In addition, an increase in conjugated BAs in serum has been associated with NASH.[188–191] However, Puri *et al*.[189] reported an increase of serum levels of total primary BAs and a decrease of total secondary BAs in NASH patients, while Jiao *et al*.[187] described the opposite trend. Regarding specific BA species, Jiao *et al*. described a decrease of CDCA and an increase of ~4 fold of DCA in the serum levels of NASH patients. A similar trend has also been reported by Puri *et al*,[189] although the differences were not statistically significant. This increase in DCA versus CDCA could be relevant for gut barrier function since DCA counteracts and inhibits the agonistic activity of CDCA in FXR,[192] the receptor shown to be involved in host antibacterial defense. In addition, DCA has been shown to directly affect IECs in mice models increasing cell proliferation, colonic nuclear damage and colonic tumor incidence.[193] However, DCA has also been shown to induce mucin production in colon cancer cells, increasing MUC2 transcription via modulation of NF-κβ and JNK,[194] suggesting it as a common mechanism for cytoprotection *in vivo*. These results manifest the complex interaction of BAs with the host in general and with gut barrier integrity in particular.

In a recent study, worsening fibrosis severity in non-obese NAFLD patients has been correlated with an increase in the synthesis of bile acids. The same study reported a depletion of *Ruminococcaceae* in non-obese subjects with significant fibrosis and, hence, elevated BA synthesis.[195] *Ruminococcaceae* has been described as a contributor to the *bsh* gene, implicated in the metabolism of BAs; which was shown to be downregulated in the same subjects.[195] The elevated synthesis of BAs and the previously mentioned higher sensitivity of Gram-positive bacteria to them could explain the decrease in the *Ruminococcaceae* clade. In contrast, Adams *et al*.[196] associated a higher abundance of DCA with the abundance of the *Lachnospiraceae* family and advanced liver fibrosis. The increase in abundance of the *Lachnospiraceae* family could explain the increase in DCA levels since this clade has been shown to have bile acid-inducible 7α-dehydroxylation genes.[182]

With regard to GI tract microbiome signatures in the disease state, Jiao *et al*. described that two BA-related pathways were elevated in NASH patients: "glycine, serine and threonine metabolism" and "taurine and hypotaurine metabolism".[187] In addition, they described a ~7.2 fold increase in the abundance of bacteria able to metabolize taurine, mainly explained by an increase in the genera *Escherichia* and *Bilophila*. It is important to note that taurine has recently been reported to improve GI tract barrier function via enhancing the expression of ZO-1 and occludin in Caco-2 cells.[97] Moreover, Ahmadi *et al*.[97] showed how the treatment with a human-origin probiotic cocktail, composed by 5 *Lactobacillus* and 5 *Enterococcus* strains, is able to ameliorate leaky gut in older mice via modulating the microbiota-taurine-TJs axis. The probiotic cocktail was shown to enhance BSH activity, releasing a higher quantity of taurine, reducing GI tract leakiness and demonstrating the importance of microbial activity in host physiology.

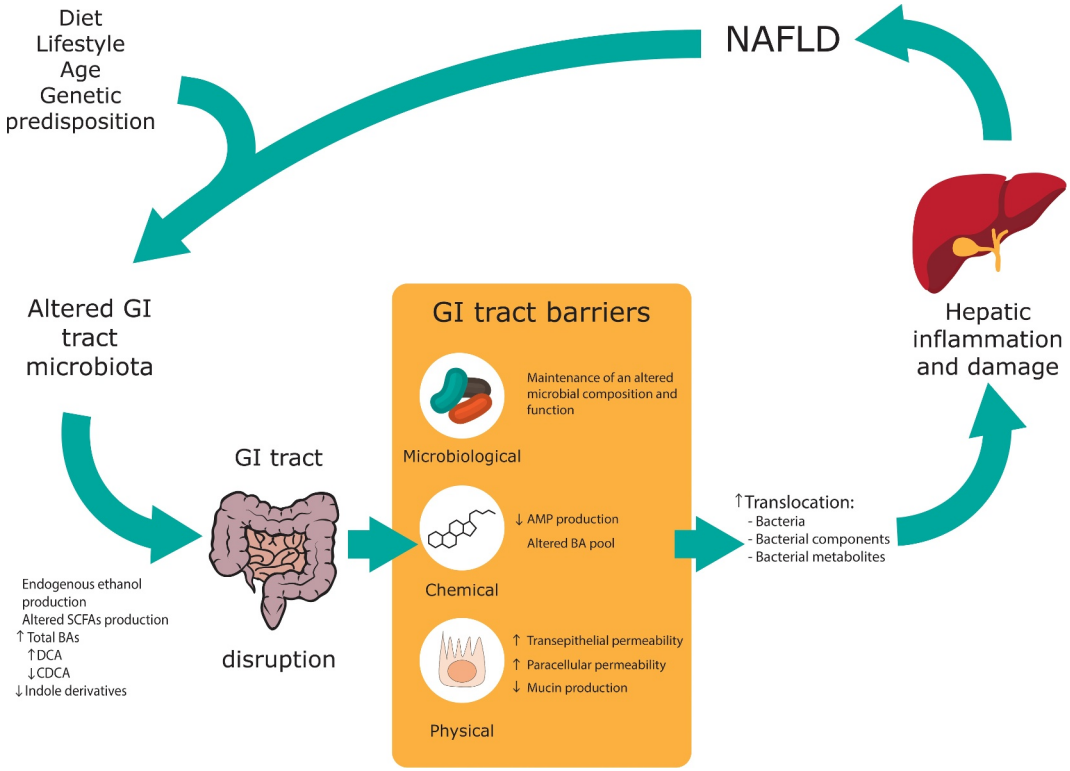

Figure 2. Alterations in gastrointestinal (GI) tract barriers associated with nonalcoholic fatty liver disease (NAFLD) pathogenesis. An altered GI tract microbiota due to diet, lifestyle, age and genetic predisposition can lead to an altered pool of metabolites along the GI tract. An altered microbiota and metabolite composition can lead to GI tract barriers disruption, with a special focus in this review to the microbiological, chemical and physical barriers. The increase in gut permeability leads to an incremented translocation of bacteria, bacterial components and bacterial metabolites to the portal system; which ultimately cause hepatic inflammation and damage and, hence, contributing to NAFLD pathogenesis. SCFA: Short-chain fatty acid, BA: Bile acid, DCA: Deoxycholic acid, CDCA: Chenodeoxycholic acid; AMP: Antimicrobial peptides.

Nevertheless, it is important to note that the pool of BAs and their effect on the host are different between mice and humans. Mice, besides CDCA and CA, also produce muricholic acids (MCAs) as primary BAs. Tauro-βMCA, not present in humans, is considered an antagonist of FXR and, consequently, deconjugation thus promotes FXR signaling in mice.[197] This difference in BA signaling between mice and human models points out the importance of being cautious when extrapolating results from mice to human models.

4.4. Tryptophan

Evidence in mice models and in clinical studies indicate that tryptophan (Trp) metabolism is involved in NAFLD progression (Supplementary Table 2, Figure 2).[198,199] Trp is an important amino acid present in food and once it is delivered into the GI tract, it is mainly involved in three pathways: 1) the direct transformation of Trp into several different derivatives by the gut microbiota (represents the 4–6% of ingested Trp); 2) the kynurenine (Kyn) pathway (KP) (95% of ingested Trp) via indoleamine 2,3-dioxygenase (IDO1); and 3) the serotonin (5 hydroxytryptamine [5HT]) production pathway (1–2% of ingested Trp) via Trp hydroxylase 1 (TpH1).[7]

Commensal bacteria are able to metabolize tryptophan into several different derivatives, such as indole pyruvic acid and indole acetaldehyde. The indole pyruvic acid can be transformed into indole propionic acid (IPA); meanwhile, the indole acetaldehyde can be transformed to indole acetic acid (IAA) and then in indole aldehyde (IAld).[7] In healthy intestine indoles have been shown to be aryl hydrocarbon receptor (AHR) ligands promoting IL-22 production which controls epithelial cell proliferation, AMPs production[200-202] and are involved in the expression of mucin genes.[203] Moreover, indole attenuates inflammation, reducing the levels of TNF-α and IL-8, and increasing the levels of the expression of the

anti-inflammatory IL-10 in the human enterocyte cell line. Venkatesh *et al.*[204] have shown in mouse models that IPA promotes intestinal barrier integrity upregulating the expression of ZO-1, occludin and E-cadherin through the activation of pregnane X receptor (PXR).[204] Therefore, indoles in NAFLD could be a player for promoting gut barrier function and it deserves more in-depth studies.

Most of the tryptophan ingested (95%) is transformed into Kyn by IDO1 in the gut. Laurans *et al.*[205] have shown increased IDO1 activity in high-fat diet mouse models and decreased indole derivatives and deletion of IDO1 increases intestinal availability of indoles. They also confirmed a shift in Trp metabolism toward more Kyn production and less indole derivatives in feces of subjects with metabolic syndromes.[205] Overexpression of the Kyn pathway has also been shown in NAFLD subjects.[206] Changes in the balance of Trp metabolism, a decrease of indoles production and an increase of Kyn might be involved in GI barrier functions in NAFLD pathogenesis but no evidence has been shown yet.

While a small amount of ingested Trp is involved in 5HT production, more than 90% of the body's 5HT available is produced in the GI tract by the enzyme TpH1 in enterochromaffin cells.[207] A consistent amount of 5HT production is regulated by the gut microbiota, especially by spore-forming bacteria that stimulate ECs to produce it in the mouse colon but not in the small intestine.[208] Moreover, the probiotic *Escherichia coli* Nissle *1917* has been shown to affect the 5HT production in *ex vivo* mouse ileum experiments, increasing the 5HT availability.[209] Recently, Fung *et al.*[210] have shown that the crosstalk between 5HT and microbiota is bidirectional. In fact, *in vitro* experiments, the authors have observed that *Turicibacter* is able to sense, uptake and respond to 5-HT regulating its colonization in the GI tract.[210]

5HT can exert its functions through a high number of receptors widely expressed in the intestine.[207] Interestingly, evidence of a correlation between 5HT and NASH has been recently shown in serum levels of NAFLD patients and in serum levels and duodenum of rat fed with high fat-sucrose diet inducing NASH.[211] The inhibition of 5HT in those rats and in *in vitro* models of NAFLD alleviates hepatic lipid accumulation and inflammation.[211] The detrimental role of 5HT in NAFLD has also been shown in *Tph1* knockout mice.[212,213] where 5HT is able to upregulate lipogenic genes in the liver through hepatic serotonin receptor HTR2A.[213] Moreover, the inhibition of the 5-HT3 receptor in mice fed fructose has shown to alleviate hepatic steatosis.[214] Whether 5HT is linked with gut barrier permeability observed in NAFLD is still unclear.

Some studies have linked 5HT with the regulation of intestinal permeability. 5HT has been shown to promote the translocation of LPS in the ileum of rats via the 5HT3 receptor.[215] In accordance, 5HT reinforced the permeability induced by NaCl perfusion in the duodenum of rats. Moreover, the use of 5HT3 receptor antagonists (granisetron and ondansetron) and 5HT4 receptor antagonist (SB 203186) restored the duodenal mucosal barrier integrity.[216] Huab *et al.*[214] have observed with an *in vitro* model, that 5HT treatment decreased significantly the TJ occludin expression in a dose-dependent way.[214] The same group has also shown that leptin-deficient obese mice treated with antagonists (ropisetron and palonosetron) of the 5HT3 receptor have increased expression of occludin, and claudin-1 in the duodenum.[217]

The mechanisms governing the 5-HT effects in the gut barrier through its receptors are not fully understood yet, as well as its crosstalk with the gut microbiota. 5HT represents a potentially interesting candidate of gut barrier dysfunction observed in NAFLD and deserves more investigation.

5. Conclusions

Based on the currently existing knowledge, we have reviewed 1) how in NAFLD the gut barrier functions are impaired, 2) how bacterial communities differ in NAFLD and NAFLD-related fibrosis and cirrhosis, 3) how specific metabolites vary in NAFLD, and 4) how microbiota and these related metabolites might have an impact in gut barrier damage.

The GI tract contains several barriers playing essential roles in maintaining the GI tract homeostasis. In human clinical studies, GI tract barrier dysfunctions observed in NAFLD are usually assessed by analyzing TJs expression in biopsies from the small intestine. An invasive and challenging method,

which, on the other hand, does not take into account the characteristics of the different intestinal barriers and the crosstalk between them along the GI tract. It is, therefore, expected that the gut barrier functions and permeability characteristics may differ in the different segments of the GI tract. It is relevant to point out that gut barrier dysfunction studies are mainly focused on the alteration of the microbiological and physical barriers leaving out the potentially important role of the chemical and immunological barriers and the recently described GVB.

Several metabolites have been shown to modulate gut barrier integrity. Some of the described metabolites, including ethanol, acetaldehyde and some BAs such as DCA, have been associated with impairment of gut permeability. While others, such as the SCFA butyrate and indoles derived from tryptophan metabolism, have been shown to promote gut barrier integrity. However, there is a lack of consensus about the role of some of these metabolites regarding gut permeability modulation, including the mentioned "butyrate paradox". These contradictory results could be a consequence of differences in study designs, but also of the complexity of the system in which these metabolites exert their action.

Different bacterial taxa have been identified to influence the presence and abundance of the metabolites. Alterations in the GI tract microbiota, as have been shown in NAFLD, will be translated into an altered pool of metabolites and, hence, affect the gut barrier function (Figure 2). Even more, these alterations in the GI tract microbiota will also shape interactions between different BGM taxa. For instance, increased endogenous ethanol production, primary BA deconjugation or AMP secretion could subject neighboring bacteria to different ecological pressures for which particular taxa might be maladapted and then outcompeted. Indeed, the antimicrobial potential of these bacteria cannot be neglected: they play a major role in shaping the communities and keeping a good balance for the optimal metabolic activity. This topic would need increased attention in future studies on the positive effects some species have to avoid NAFLD disease. However, the most common sampling method for microbiota analysis, fecal sampling, is not able to reflect the community structure of all the different intestinal segments. Therefore, potential relevant processes and ecological transitions are lost. Current technology often fails to detect bacterial taxa at the strain level. Instead, abundance is generally referred to as genus, family or even phylum level, overlooking the impact an individual strain could have. Another essential aspect to consider is that the gut microbiota is composed not only by bacteria but also by fungi, archaea, protists and viruses. However, most of the studies linking gut microbiota alterations with NAFLD pathogenesis refer solely to bacteria. More research focused on unraveling the role of under-represented micro-organisms in the microbiota is of crucial importance.

How the bacterial gut microbiota influences, partly via produced metabolites, the disruption of the gut barrier; what the influence in this process is of other gut microbes; and how the crosstalk between the host and its microbiota intervenes in NAFLD pathogenesis are still unresolved questions. Consequently, more studies reflecting the complexity of the crosstalk between GI tract microbiota, host and metabolites are required to disentangle their causal effect in modulating gut permeability in NAFLD pathogenesis.

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