



REVIEW

Gut–liver axis: The impact of gut microbiota on non alcoholic fatty liver disease

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Abstract *Aim:* To examine the impact of gut microbiota on non alcoholic fatty liver disease (NAFLD) pathogenesis.

Data synthesis: Emerging evidence suggests a strong interaction between gut microbiota and liver. Receiving approximately 70% of its blood supply from the intestine, the liver represents the first line of defence against gut-derived antigens. Intestinal bacteria play a key role in the maintenance of gut–liver axis health. Disturbances in the homeostasis between bacteria- and host-derived signals at the epithelial level lead to a break in intestinal barrier function and may foster “bacterial translocation”, defined as the migration of bacteria or bacterial products from the intestinal lumen to mesenteric lymph nodes or other extraintestinal organs and sites. While the full repertoire of gut-derived microbial products that reach the liver in health and disease has yet to be explored, the levels of bacterial lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver diseases. Derangement of the gut flora, particularly small intestinal bacterial overgrowth, occurs in a large percentage (20–75%) of patients with chronic liver disease. In addition, evidence implicating the gut–liver axis in the pathogenesis of metabolic liver disorders has accumulated over the past ten years.

Conclusions: Complex metabolic diseases are the product of multiple perturbations under the influence of triggering factors such as gut microbiota and diet, thus, modulation of the gut microbiota may represent a new way to treat or prevent NAFLD.

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Introduction

A close interplay exists between the gut and liver, named “gut–liver axis”. This relation has been based on the evidence that beneficial substances produced by the liver are absorbed by the gut and more than 70% of the blood liver supply derives from the portal vein, the direct venous outflow of the intestine [1]. An impaired gut barrier exposes the liver to gut-derived toxic factors, and a disrupted liver physiology may prompt gut dysfunction. A key role in the maintenance of gut–liver axis health has been attributed to intestinal bacteria.

The gastrointestinal tract, particularly the large bowel, contains the largest number of bacteria of the human body. Recent data estimate 10–100 trillion microorganisms and at least 500–1000 different species [2]. The intestinal microflora consists of a dynamic mixture of microbes that quantitatively and qualitatively greatly differ among species and individuals [3]. In addition, surface-adherent and luminal microbial populations also differ, indeed, the ratio of anaerobes to aerobes is lower at the mucosal surfaces than in the lumen [4]. Dietary habits, lifestyle, age, host genotype and exposure to antibiotics also may affect the composition of the gut microflora [5]. Humans and intestinal bacteria have developed an adaptive commensal relationship supported by the synergistic interplay of multiple intestinal defence mechanisms, including luminal factors, inhibition of mucosal attachment, prevention against penetration and immunological clearance mechanisms.

Here we examine the impact of gut microbiota on liver diseases, focussing on non alcoholic fatty liver disease (NAFLD).

Gut microbiota

The intestinal mucosa is the main interface between the immune system and the external environment. The dialogue between host and bacteria at the mucosal interface seems to play a part in the development of a competent immune system. The gut microbiota elicits innate and adaptive immune mechanisms that cooperate to protect the host and maintain intestinal homeostasis. Activation of innate host defence depends on specific pattern recognition receptors (PRRs) that recognize highly conserved microbial signature molecules called “pathogen-associated molecular patterns” (PAMPs). The PRRs include the family of toll-like receptors (TLRs) and NOD-like receptors (NLRs) [6]. Different TLRs recognize different classes of PAMPs, which are specific for different bacterial species. Thus far, eleven TLRs have been identified in humans, among which TLRs 2, 4 and 9 are the most relevant in Gram-positive and Gram-negative bacteria recognition and signalling [6]. Stimulation of TLRs by PAMPs leads to the activation of nuclear factor kappa B (NFkB) that, in turn, results in the transcription of inflammatory cytokines, chemokines and antimicrobial genes [7].

The gut mucosal arm of the adaptive immune system, localized predominantly in the small bowel, provides humoral and cell-mediated immunity against ingested antigens and luminal organisms. The gut-mucosal immune

system consists of gut-associated lymphoid tissue (GALT), the largest immunological organ of the body, which comprises four lymphoid compartments: Peyer’s patches, lamina propria lymphocytes (including dendritic cells), intra-epithelial lymphocytes and mesenteric lymph nodes. Activation of the mucosal immune system produces mucosal and serum antibody responses, T cell-mediated immunity and local immunostimulatory or immunosuppressive mediators [8].

The complex interaction among bacteria, epithelium and gut immune system is a prerequisite for the development of mature immune functions and defence mechanisms in the gut. Studies of animals bred under germ-free conditions show that germ-free animals display morphological, structural and functional abnormalities (reduced vascularity, digestive enzyme activity, muscle wall thickness, cytokine production and serum immunoglobulin levels, smaller Peyer’s patches and fewer intra-epithelial lymphocytes) [9]. Interestingly, reconstitution of germ-free mice with an intestinal microflora, such as *Bacteroides thetaiotaomicron*, is sufficient to restore the mucosal immune system [10].

The continuous and intimate contact between gut bacteria and mucosal surface implies that indigenous microbes profoundly influence neighbouring host cell functions. Consistent with this prediction, a growing body of experimental evidence reveals that luminal bacteria drive key epithelial cell functions that help to maintain barrier integrity.

Major functions of the gut microflora include metabolic activities, trophic effects, immunity and protection of the colonised host against invasion by alien microbes [11]. A prominent metabolic function of gut microflora is the fermentation of non-digestible dietary residue. The end products of this complex metabolic activity are a spectrum of organic acids that are an important energy source for the host (5–15% of human energy requirement). Fermentation of carbohydrates (large polysaccharides, some oligosaccharides that escape digestion, and unabsorbed sugars and alcohols) generates short-chain fatty acids that influence cell differentiation and proliferation, ion absorption (Ca, Mg, Fe) and the vitamin production. Proteolytic fermentation generates polyphenols that induce anti-inflammatory, anti-oxidative and anti-ageing effects. Anaerobic metabolism of peptides and proteins (putrefaction) by the microflora produces a series of potentially toxic substances including ammonia, amines, phenols, thiols, and indols [12].

Another major function of the intestinal microflora is protection against exogenous microorganisms. Adherent non-pathogenic bacteria can inhibit the growth of pathogenic bacteria through the synthesis of antimicrobial substances or by nutrient competition and prevent attachment and penetration of pathogen enteroinvasive bacteria in the epithelial cells [13].

The microbiota itself forms an integral part of the natural mechanisms that safeguard the barrier integrity. Disturbances in the homeostasis between bacteria- and host-derived signals at the epithelial cell level lead to a break in the intestinal barrier and may foster “bacterial translocation”, defined as migration of bacteria or bacterial products from intestinal lumen to mesenteric lymph nodes or other extraintestinal organs and sites [13].

In summary, the gut microbiota, by regulating metabolic, trophic, immune and barrier function, protects the host against digestive and extra-digestive diseases.

Gut–liver axis

The occurrence of a cross-talk between the gut and the liver is an intriguing hypothesis that could account for the hepatobiliary alterations observed in several inflammatory and infectious intestinal diseases. The liver, receiving most of its blood supply from the intestine through the portal vein, and because of its rich supply of blood sinusoids, is one of the organs most exposed to gut-derived toxic factors, including bacteria and bacterial products. The intestinal microflora produces ethanol, ammonia and acetaldehyde that are generally metabolized by the liver and are able to control Kupffer cell activity and cytokine production [14]. In addition, quantitative and qualitative variations of intestinal bacteria result in an increase in intestinal permeability and endotoxin translocation that, in turn, induces the transcriptional activation of a wide variety of proinflammatory genes and cytokines in the liver [15]. While the full repertoire of gut-derived microbial products that reach the liver in health and disease has yet to be explored, the levels of bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver diseases [16].

To counteract the effects of bacterial translocation, the liver contains a large number of immune cells of both the innate and adaptive immune system which participate in both tolerance and inflammation within the liver. Bacteria and bacterial products induce activation of Kupffer cells, the macrophages of the liver. Kupffer cells, through a NFkB-mediated mechanism, trigger the release of cytokines such as tumour necrosis factor alpha (TNF α) that may activate specific intracellular pathways, i.e., pro-apoptotic signals via the caspase cascade [17]. The exposure of the liver to LPS, that behaves as a hepatotoxin, is associated with morphological and functional changes that induce an acute inflammatory response with early accumulation of polymorphonuclear cells. Neutrophils release reactive oxygen metabolites, proteases and other enzymes from the granules, with a worsening of the liver damage [18].

Gut microflora and minimal liver damage: metabolic liver disorders

Gut microflora was first found to be altered in patients with chronic liver disease more than 80 years ago. Derangement of the gut flora, in particular small intestinal bacterial overgrowth (SIBO), occurs in a large percentage (20–75%) of patients with chronic liver disease.

NAFLD includes steatosis and non alcoholic steatohepatitis (NASH, characterized by steatosis and periportal and lobular inflammation). Two types of NASH have been described: primary NASH (associated with metabolic syndrome-related conditions i.e., obesity, type 2 diabetes and hyperlipaemia) [19–21] and secondary NASH (occurring after jejuno-ileal bypass surgery, rapid weight loss, total

parenteral nutrition, drugs, lipodystrophy or Wilson's disease). Steatosis has been reported in up to 95% of obese patients and NASH in up to 20% of obese patients [22]. However, the pathogenesis of NASH is still not fully understood.

According to the classical theory, NASH develops in two steps: first the healthy liver become steatotic as a consequence of insulin resistance that, in turn, increases the transport of fatty acids from adipose tissue; second, additional insults, such as bacterial LPS, induce oxidative stress and production of cytokines, particularly TNF α , that sustain liver damage [23]. A recent theory suggests that, because simple hepatic steatosis is a benign process in the majority of patients, NASH might be a separate disease with a different pathogenesis. Many hits, especially gut-derived and adipose tissue-derived factors, may act in parallel and finally result in liver inflammation [24].

Evidence supporting a role for the gut–liver axis in the pathogenesis of NASH has slowly accumulated over the past ten years [25]. First NASH was encountered as a common complication of jejuno-ileal bypass surgery for morbid obesity. Moreover, NASH has been reported in individuals with jejunal diverticulosis and intestinal bacterial overgrowth. Finally various rat models of intestinal bacterial overgrowth have been associated with liver lesions similar to NASH. The pathogenetic role of intestinal bacteria is also supported by the observation that the administration of antibiotics, improved steatosis in both rats and humans on total parenteral nutrition, following intestinal bypass surgery and in alcohol exposed rats [26].

The gut bacteria may contribute to the pathogenesis of NASH through three mechanisms: 1) increase in luminal gut ethanol production; 2) metabolism of dietary choline (required for very-low-density lipoprotein synthesis and hepatic lipid export); and 3) release of LPS. Both ethanol and LPS stimulate inflammatory cytokine production through an NFkB-mediated mechanism.

SIBO is reported in patients with NAFLD and has been associated with the severity of steatosis [14,27]. Although, the pathogenetic link between SIBO and NASH development and progression has not yet fully elucidated, intestinal permeability seems to play a key role. Thuy et al. recently found that endotoxin and plasminogen activator inhibitor (PAI)-1 plasma levels, as well as liver expression of TLR4 and PAI-1 mRNA were significantly higher in NAFLD patients than in controls [28]. In addition, Miele et al., very recently reported that NAFLD in humans is associated with increased gut permeability and increased prevalence of SIBO [29]. In support of this pathogenetic implication, the administration of antibiotics, such as polymyxin B and metronidazole, has been proven to reduce the severity of steatosis in both animal models and man [30,31].

Recently, the gut microbiota has been shown to affect fat storage and energy harvesting, thereby playing a direct role in the development of insulin resistance and related metabolic diseases [32–34]. Obese humans harbour considerably fewer Bacteroidetes and more Firmicutes than lean controls and recent evidence suggest that gut microbiota directly affects the proportion of calories obtained from the intestinal contents [35].

Depending on specific dietary conditions, the intestinal microflora changes lead to increased intestinal permeability

and consequent endotoxemia that in turn triggers inflammation and metabolic disorders. In animal models, high fat feeding affects bacterial populations, favouring an increase in the Gram-negative/Gram-positive ratio and promoting endotoxemia. Very recently, a metagenomic analysis of the microbial communities living in the intestinal tracts of 15 women at choline-depleted diet, revealed a strong correlation between the relative abundance of two specific classes of bacteria, *Gammaproteobacteria* and *Erysipelotrichi*, and development of fatty liver [36].

Endogenous LPS is transported from the intestine toward target tissues by a mechanism facilitated by chylomicrons synthesized in response to a high-fat diet, and triggers the secretion of pro-inflammatory cytokines when it binds to specific TLRs. It has been shown that TLR2, TLR4, and TLR9 play a role in the development of NASH [37,38]. TLR4-mutant mice display decreased injury and lipid accumulation following a methionine-choline-deficient (MCD) diet, a well known model of NASH [39]. In contrast, MCD diet induced NASH in TLR2-deficient mice [40]. The role of TLR4 signalling in NASH is further emphasised by the ability of the TLR4 ligand LPS but not the TLR2 ligand peptidoglycan to exacerbate liver injury in mice treated with an MCD diet. Miura et al. have recently identified TLR9, which recognizes bacterial unmethylated CpG DNA, as another important player in the pathogenesis of NASH based on a murine model of NASH induced by a choline-deficient amino acid-defined diet [41]. To date, few data are available regarding the role of TLRs in NASH pathogenesis in humans. Our preliminary data, obtained by quantitative real time PCR on liver biopsies, show significantly higher TLR4 and lower TLR2 expression in patients with NASH than in patients with steatosis or control subjects (Fig. 1). Studies

aimed at understanding the TLR4, TLR2 and TLR9 signalling in NASH may help us to design novel therapeutic strategies to treat this disorder.

Taken together, these findings strongly support the idea that complex metabolic diseases are a product of multiple perturbations under the influence of triggering factors such as gut microbiota and dietary habit.

Gut microbiota modulation as an emerging treatment strategy

A scarcity of effective treatments exists for the management of NAFLD. The role of gut microbiota in the pathogenesis of the disease opens the door to new ways of thinking about NASH prevention and treatment.

Probiotics are defined as viable microorganisms that, when administered in adequate amounts, confer a health benefit to the host [42]. There are many mechanisms by means of which probiotics positively affect the gut microbiota and liver health, i.e., inhibition of intestinal bacterial enzymes, stimulation of host immunity, competition for limited nutrients, inhibition of bacteria mucosal adherence and epithelial invasion, protection of intestinal permeability and control of bacterial translocation from the gut to the bloodstream. The biological activity of probiotics depends prevalently on delivering anti-inflammatory mediators that down-regulate pro-inflammatory cytokines, including IFN- γ and TNF α , via the NF κ B pathway [43]. Therefore, probiotic therapy offers an intriguing approach to control hepatic cellular stress and promote host health.

Several animal models have provided evidence that probiotic supplementation may affect NASH development and progression (Table 1) [44–48]. In *ob/ob* mice, modifications of the gut microbiota following probiotic administration improved hepatic insulin sensitivity, hepatic steatosis and inflammation. *In vivo* administration of VSL#3, a probiotic preparation of 8 different live, frozen-dried bacteria, had a beneficial effect on liver steatosis in *ob/ob* mice [47]. We recently found that dietary supplementation with *Lactobacillus paracasei* F19, by restoring the gut microflora and intestinal barrier function, as shown by decreased LPS levels, attenuated oxidative and metabolic liver damage [48]. A recent randomized clinical study performed on patients with histologically proven NAFLD showed that supplementation with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* improved liver aminotransferases levels [49].

The possibility to modulate gut microbiota to treat NAFLD has aroused, in recent years, growing interest in the potential for prebiotics as an effective dietary treatment. Prebiotics are defined as 'a non-digestible food ingredient that beneficially affects the host, by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon' [50]. Several mechanisms have been proposed to explain the beneficial effects of prebiotic fibres on serum lipids and the accumulation of triglycerides in the liver observed in animals, including reduced de novo fatty acid synthesis and short-chain fatty acid production, body weight and fat loss, improved glycaemic control, microbial modulation and reduced inflammation. Parnell et al., through a systematic review of animal model studies, showed that prebiotic supplementation positively impacts

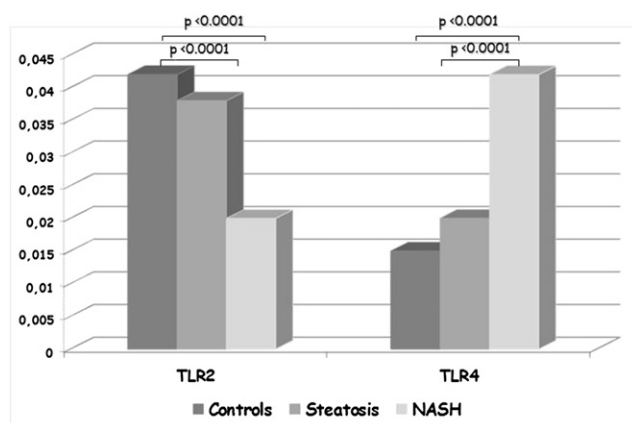


Figure 1 Quantitative real time PCR for Tool-like receptors (TLR) 2 and 4 on liver tissue samples from patients with steatosis, non alcoholic steatohepatitis (NASH) and control subjects. Twenty-three subjects with abdominal ultrasound evidence of steatosis and history of mild-moderate hypertransaminasemia by at least six months, underwent liver biopsy through which they were classified as affected by steatosis (*n*. 13) and steatohepatitis (*n*. 10). Control subjects (*n*. 11) were recruited among those who underwent laparoscopic cholecystectomy due to gallstone disease, and performed intra-operative liver biopsy in the absence of metabolic disturbances or ultrasound evidence of steatosis.

Table 1 Animal interventional studies on probiotics and metabolic liver diseases.

Author	Year	Animal model	Treatment	Results
Li Z [44]	2003	<i>Ob/Ob</i> mice	VSL#3	↓ Total hepatic fat ↓ ALT ↓ TNF α RNA
Ewaschuk J [45]	2007	129Sv/Ev WT + LPS 129Sv/Ev IL-10 ⁻ + LPS		↓ Liver damage ↓ Intestinal damage
Ma X [46]	2008	C57BL6 mice	VSL#3	↓ Liver NK cells ↓ TNF α IL-4
Velayudham A [47]	2009	C57BL6 mice C57BL6 mice + MCD diet	VSL#3	↓ TGF β receptor ↓ Liver fibrosis
Nardone G [48]	2010	Winstar rats	<i>Lactobacillus paracasei</i> F19	↓ TNF α , IL-1 β , IL-6 RNA ↓ Serum LPS ↓ Liver inflammation, steatosis and fibrosis

NAFLD by modifying the gut microbiota, reducing body fat, and improving gluoregulation, thus representing an attractive therapeutic strategy [50].

Unfortunately, no studies have addressed the effect of antibiotics; however, the positive effect of polymixin B and metronidazole in reducing the severity of steatosis during total parenteral nutrition or after intestinal bypass, in both animal models and man, provides a reasonable prospect for the use of antibiotics to treat NAFLD [30,31].

Overall, until now there are only few data concerning the use of probiotics, prebiotics and antibiotics in humans, therefore, large-scale randomized controlled trials, with histological endpoints are needed.

Conclusions

Gut microbiota contribute to normal intestinal epithelial cell biology and function. In the absence of appropriate immune cell regulation or when gut barrier function is impaired, gut bacteria may contribute significantly to various acute and chronic liver diseases by activating the innate and adaptive immune responses and wound healing processes. Thus, modulation of the gut microbiota may represent a new way to treat or prevent a variety of liver diseases. However, several questions remain unanswered, namely (1) which are the specific populations of the gut bacteria responsible for liver damage? (2) can these bacterial populations be correlated with or used as a screening tool for the progression of liver disease? (3) how do different microbiota populations influence gut barrier integrity? and (4) which is the specific immune reaction underlying the gut microbiota-related liver diseases? Studies designed to address these questions will provide information about the connection between the gut microbiota and liver disease and will probably lead to new therapies for liver disease or predictors of liver pathobiology.

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