Gut microbiota and liver disease
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Abstract
Microbes are present in large numbers in each human being, in particularly in the gastrointestinal (GI) tract, and have long been believed to have some beneficial effects for their hosts. Till recently, however, we lacked tools for studying these organisms. Rapid technological advances in recent years have markedly improved our understanding of their role both in health and disease. Recent literature suggests that organisms in the GI tract, referred to collectively as gut microbiota, play an indispensable role in the maintenance of host’s homeostasis. Alterations in the gut microbiota, that is in the nature and relative density of various constituent bacterial species, appear to have a role in pathogenesis and progression of several GI and hepatic diseases. This has also opened the vista for tinkering with gut flora in an attempt to treat or prevent such diseases. In this review, we have tried to summarize information on normal gut microbiota, laboratory techniques and animal models used to study it, and the role of its perturbations in some of the common hepatic disorders, such as non-alcoholic fatty liver disease (including obesity), non-alcoholic steatohepatitis, alcoholic liver disease, and liver cirrhosis and its complications.

Introduction
The human body, instead of being a single organism, is actually a complex ecosystem comprising of fauna representing all three major domains of life, namely bacteria, archaea, and eukaryotes. This is because its various surfaces, such as skin, oral cavity, vaginal mucosa, respiratory passages, and, most importantly, the gastrointestinal (GI) tract are colonized by a wide variety of microorganisms. These surfaces provide a favorable habitat for these organisms to reside and thrive.

The term “gut microbiota” refers to a complex mixture of diverse microbes present in the GI lumen of an individual. It consists of approximately $10^{14}$ microbial cells, that is a number nearly 10-fold larger than that of human cells in an adult. Density, diversity, and relative composition of bacterial species vary along the length of the GI tract, being the most numerous in oral cavity and colon. Acidic environment in the stomach and rapid motility of the small intestine ensure that bacterial density in these organs is very low. In contrast, colonic contents contain nearly $10^{11}$–$10^{12}$ bacteria per gram of feces, with obligate anaerobes dominating over aerobes and facultative anaerobes in a ratio of 100–1000:1.

Nearly 99% of microbes in the human gut belong to 1000–1200 bacterial species, with the remainder being contributed by archaea, viruses, and prokaryotes. These bacterial species belong mostly to genera that are placed in one of the four phyla, namely Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, with little representation from the other bacterial phyla.

Key words
alcoholic liver disease, encephalopathy, gastroenterology, hepatology, microbial pathogenesis, NAFLD, NASH, pathogenesis, portal hypertension.

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flows to the gut to affect the abundance and distribution of various organisms in the latter’s lumen. This close relationship of liver and gut implies that gut flora may play a role in the pathogenesis of liver diseases, and their study may allow identification of newer preventive and therapeutic strategies against these diseases.

This article reviews in brief the techniques used to study gut microbiota and current knowledge about the role of microbiota in liver disease.

**Techniques for studying gut microbiota**

Initial studies on composition of gut microbiota were based on culture of intestinal biopsy specimens, luminal contents, or feces. In recent years, these have largely been replaced by molecular methods.

**Culture-based methods.** These methods use microbiological culture techniques in which a diluted specimen is plated on a non-selective, selective, or enriched medium that supports the growth of one or more bacterial types or groups. The bacterial colonies are then identified based on morphological and biochemical characteristics. These techniques are simple, cheap, readily available, and allow specific detection and semiquantitative estimation of several bacterial groups such as *Bacteroides* spp., *Eubacterium* spp., *Bifidobacterium* spp., *Lactobacilli*, and *Clostridium* spp.

However, several bacterial species, in particular strict anaerobes, are quite fastidious, and do not grow on the available culture media under usual laboratory conditions. Data from newer molecular methods indicate that culture methods cannot detect nearly 80% of the bacterial species resident in the human gut and thus underestimate the diversity of gut flora.\(^7\) On the contrary, different strains of the same bacterial species may at times vary in their characteristics, providing a false sense of diversity.\(^8\)

**Culture-independent molecular methods (Table 1).** These techniques depend on diversity in the sequence of the bacterial 16S ribosomal RNA (rRNA) gene, which is present in all bacteria. It is 1.5 kilobase in length and has some regions that are strongly conserved and others that are highly variable. This provides an appropriate balance of conservation among larger phylogenetic groups and sufficient variability to distinguish between different species.\(^9\) Several techniques, each with its own advantages and limitations, have been developed to exploit this variability in 16S rRNA gene for the study of gut microbiota.

**Full-length 16S rRNA sequencing.** In this technique, the entire 16S rRNA gene is amplified and sequenced; the bacterial species is then determined by comparing the sequence against one of several databases that contain sequences of this gene in various organisms. In recent years, these have largely been replaced by molecular methods.

| Technique | Comments |
|-----------|----------|
| Culture-based techniques | Simple and cheap. May fail to detect about 80% of bacterial species present in the gut that cannot be cultured |
| Culture-independent DNA-based techniques | Molecular-based techniques that use variation in bacterial 16S rRNA gene to identify the bacterial group or species; can identify majority of gut microbes with high specificity |
| Sequencing of 16S ribosomal RNA (rRNA) gene | Provides accurate species identification. High cost, time consuming, not applicable to bacterial mixtures, particularly those with non-culturable bacteria |
| Pyrosequencing of 16S rRNA gene segments | Recently developed, rapid, and relatively cheaper method involving high-throughput sequencing of hypervariable segments of 16S rRNA genes; allows determination of presence and relative density of several bacteria, even in complex mixtures |
| DNA fingerprinting | Relatively rapid and less expensive than Sanger sequencing. Can generate DNA profile of bacteria in a mixture, allowing comparison of different specimens |
| DNA microarray | A quick method that can simultaneously detect several bacteria and measure the amount of each, using hybridization of 16S rRNA sequences in a bacterial mixture to a large number of specific DNA probes |
| Culture-independent techniques for studying functional activity of bacteria | Methods to study functional capability of microbes; most are currently being further developed for large-scale application |
| Metagenomics | Sequencing of entire bacterial genome, including all exons and introns, followed by bioinformatics analysis |
| Metatranscriptomics | Profiling of bacterial messenger RNAs (mRNAs), using either mRNA microarrays or sequencing of mRNA |
| Metaproteomics | Profiling of all proteins synthesized by bacteria |
| Metabolomics | Profiling of all metabolites produced by bacteria |
| Animal models for studying gut microbiota | In vivo models. Allow study of functional aspects of relationship between the host and gut bacteria |
| Germ-free (GF) animals | Animals born in and raised in sterile environment; comparison with normal animals shows effect of gut flora |
| Animals colonized with one or multiple bacterial species | GF animals colonized with one or more bacterial species; allow study of effect of colonizing bacterial species on host, and also interaction between various bacteria in those colonized with multiple bacteria |
| Gut flora-associated animals | GF animals colonized with human or normal animal fecal material; allow study of interaction between human gut flora and a non-human mammalian host (closest to human situation) |
bacterial species. This technique provides the most accurate method of bacterial identification; however, it is costly, time consuming, and applicable primarily to pure bacterial cultures. It cannot be applied to complex bacterial mixtures such as the gut microbiota, except after successful culture of bacteria or cloning of bacterial DNA into a vector, followed by sequencing of DNA from several individual colonies or of several clones; this however is very costly. Also, these techniques introduce a bias because of failure of several bacterial species to grow or of their DNAs to be cloned.

**Partial 16S rRNA sequencing using newer-generation sequencing (pyrosequencing).** Several newer-generation sequencing platforms have become available in the last few years. These instruments allow rapid, high-throughput sequencing (nearly 25 million bases at 99% or better accuracy in a single 4-h run) at a much lower cost than traditional sequencing and have a particular advantage of ability to sequence individual molecules in a mixture of several nucleic acids. These techniques target the hypervariable segments of the 16S rRNA gene. In brief, primers based on conserved regions surrounding a hypervariable region are used to amplify nucleic acids extracted from a bacterial mixture. The random DNA fragment libraries so generated are then sequenced and compared with a 16S rRNA sequence database, providing information on the number and nature of bacterial species present in the mixture as also the relative abundance of each species.

The inherent large sequencing capacity of these techniques has been further exploited by using specimen multiplexing to drive down costs. In brief, a short bar-coding nucleotide sequence, separate for each individual specimen, is appended to the primers. DNA libraries from several specimens can then be mixed before sequencing. During the analysis step, bar codes are used to disaggregate the data for individual specimens.

A major advantage of this technique is that it does not need ex vivo bacterial culture or cloning of DNA. Thus, it provides for a more robust and bias-free determination of diversity and relative abundance of bacterial species.

**Fingerprinting methods.** Several simple and rapid culture-independent DNA fingerprinting techniques have been used for identification of gut flora. In these, segments of bacterial DNA are amplified and then separated based on their lengths or nucleotide sequences. The principles underlying these techniques are described in brief below.

In terminal restriction fragment length polymorphism, a fragment of 16S rRNA gene is amplified using a radiolabeled primer, digested using a restriction endonuclease and subjected to electrophoresis. Different bacteria give different fragment patterns depending on the presence or absence of restriction sites in their DNAs.

Other techniques are based on the fact that even a minor change in nucleotide sequence of DNA can lead to marked alterations in its physical properties. In single-strand conformation polymorphism, amplified single-stranded DNA is allowed to undergo three-dimensional folding, wherein DNA molecules of similar lengths but different sequences often assume unique conformational shapes, which are associated with different migration rates on electrophoresis. Other techniques, namely denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis (TGGE), and temporal TGGE, distinguish between different bacteria based on specific melting behaviors of their amplified DNA fragments due to sequence differences.

Bacterial rRNA is encoded by two genes (16S rRNA and 23S rRNA), which are separated by an internal transcribed spacer region, which is highly variable in both length (from 150 to 1500 bases) and nucleotide sequences. Automated ribosomal intergenic spacer analysis is an advanced fingerprinting technique that uses amplification of this region followed by electrophoresis and exploits the variation in the length of this region.

Amplified fragment length polymorphism is the most accurate fingerprinting technique. It involves digestion of DNA with restriction enzymes, followed by ligation of restriction site-specific adapters to the DNA fragments. Some of these adapter-ligated fragments are then selectively amplified using primers that bind to the adapter and a few nucleotides of the original DNA sequence near the restriction site, followed by electrophoretic separation.

The DNA fingerprinting methods, although technically less demanding and cheaper than sequencing, are at best semiquantitative, pick up only large differences between bacterial genomes, and thus have lower sensitivity in assessing bacterial diversity.

**DNA microarrays.** A DNA microarray, also known as gene chip, is a large collection of microscopic DNA spots attached to a solid surface such as glass or silicon chip. Each DNA spot contains a few picomoles (10^{-12} moles) of a small DNA, known as a “probe,” with nucleotide sequence that is specific for the DNA sequence of a particular bacterium. The probes on the chip are hybridized with DNA extracted from the test specimen, which has then been labeled with a fluorescent substance. An image of the chip is then analyzed to identify the probes have bound the labeled nucleic acids and the amount of such binding, providing semiquantitative information on the bacteria present.

The technique can detect and measure the amount of 16S rRNA for a variety of bacteria, and is cheaper and quicker than the sequencing methods, with a somewhat inferior but fairly acceptable sensitivity, selectivity, and quantification ability.

**Methods based on functional activity of bacteria.** The techniques discussed above provide information on the structure of the bacterial genome. It may instead be more important to look at characteristics of the gut bacterial community that reflect their functional abilities. This can be done through sequencing of the entire bacterial genomes including the genes encoding various bacterial enzymes (metagenomics), messenger RNA expression (metatranscriptomics), protein synthesis and composition (metaproteomics), metabolic profile (metabolomics), etc. Techniques for these are however more complex and costlier, and need further refinement before these can be used on a large scale.

**Animal models for studying gut microbiota**

Several animal models of varying complexity have been used to study the functional aspects of host–microbiota symbiosis.
Germ-free animals. Animals born and raised in a sterile environment lack gut flora, and are known as germ-free (GF) animals. A comparison of conventionally-raised animals (such as mice, pigs, and zebrafish) with their GF counterparts allows determination of the effects of gut flora on mammalian hosts. In such comparisons, GF animals have been shown to have lower fat deposits, reduced intestinal mucosal surface area, impaired bile acid and cholesterol metabolism, and impaired immune response in the intestine.1

Animals colonized with a specific bacterial group or bacterial mixture. If a bacterial species or strain is introduced into the gut of a GF animal soon after birth, it successfully colonizes the intestinal lumen. A comparison of such animals with GF animals permits inferences about interactions between the host and the particular bacterial species introduced, and more generally about the effect of presence of bacteria in the gut. Simultaneous introduction of two or more bacterial species or strains instead of one is also possible. Such simultaneous colonization provides an opportunity for studying the interactions of different bacterial species, which could arise through competition for nutrients and space, or complementary functions that the co-colonizing organisms may possess.12–14

The acquired flora in such artificially colonized animal models remains stable over a long period. Further, the animals naturally pass their flora to their progeny.15 However, these models have a relatively limited bacterial diversity than the flora in human gut.

Gut flora-associated animals. In this model, mouse or human fecal material is used to colonize the gut of GF animals. The flora in such animals also show long-term stability and are passed on to offsprings. These models are also useful in studying the alterations in gut flora and intestinal ecosystem during physiological and pharmacological interventions, such as antibiotic administration,16 circumventing the ethical issues associated with similar studies in humans. Another advantage of such models relates to avoidance of confounding by variations in human diet, lifestyle patterns, and host genetics.

Although these models more closely resemble the human situation than the mono- or bi- and poly-associated models, these may still not fully replicate the human situation. For instance, feces from humans and human flora-associated animals differ significantly in concentration of short-chain fatty acids, despite being similar in several other characteristics.17

Role of microbiota in causation of liver diseases
Liver has a dual blood supply with nearly 70% of its blood coming from intestines through the portal circulation and the remaining through the hepatic artery. Although the intestinal mucosa acts as an effective barrier against translocation of microbes and microbial products from the gut to the circulation, small quantities of these do enter the portal venous blood. Liver, being strategically located between highly contaminated bowel and sterile systemic circulation, works as a filter.

Immune cells in hepatic sinuoids effectively remove bacteria and bacterial products from the portal blood,18 protecting the systemic circulation from endotoxemia. Liver is also rich in a variety of innate immune cells, namely natural killer cells (NK cells), NK T cells, Kupffer cells, and hepatic stellate cells. These cells serve to maintain a sensitive balance between protective immune response against exogenous antigens and immune tolerance; the latter is particularly important because excessive activation of hepatic immune cells in response to exogenous antigens may induce inflammation, autoimmune phenomena, fibrosis, or carcinogenesis in the liver. Alterations in nature and number of bacterial species in the gut microflora or their increased translocation may also disturb this fine balance and lead to liver injury, particularly when “liver tolerance” to bacterial products has been breached.19

Gut microbiota have been proposed to play a role in the pathogenesis of a wide spectrum of liver diseases, such as alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH), liver cirrhosis, hepatic encephalopathy (HE), and hepatocellular carcinoma (HCC). The pathogenesis of these disease processes shows some shared features, including inflammatory cell infiltration in liver tissue, elevated serum transaminases suggesting hepatocyte damage, and increased serum levels of pro-inflammatory cytokines.

The proposed mechanisms of gut–liver interaction in these diseases (Fig. 1) include alterations in composition of gut microbiota, small intestine bacterial overgrowth, increase in permeability of small bowel, and alterations in mucosal and systemic immunity. Relationship of gut flora with liver disease may be influenced by several other factors, such as diet, toxin exposure, environmental factors, and probably genetic predisposition of an individual. Further, the alterations in microbiota may influence not only the likelihood of liver disease, but also the rate of its progression or of the development of its complications.

Fatty liver disease. Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of abnormalities, ranging from simple steatosis, characterized by excessive fat deposition in hepatocytes without any inflammation or necrosis, to NASH characterized by steatosis-associated with liver inflammation. The condition is often associated with obesity and metabolic syndrome or its individual components. A proportion of individuals with NAFLD, particularly those with NASH, progress to liver cirrhosis and portal hypertension, and may carry an increased risk of HCC.

Gut microbiota may be involved in the pathogenesis of NAFLD in several ways, namely predisposition to obesity, induction of insulin resistance, or induction of liver inflammation.20

Gut microbes and obesity. Relationship of gut flora with obesity is quite strong in mice, whose gut flora resembles that of humans in predominance of Firmicutes and Bacteroidetes. GF mice resist development of obesity when fed a high-fat, high-sugar diet.21 Introduction of gut flora in GF mice has been shown to lead to increased harvesting of energy from diet with increased intestinal monosaccharide uptake, development of insulin resistance, induction of hepatic lipogenesis and fat deposition, and increased weight and body fat content.22 More importantly, GF mice inoculated with gut flora from genetically obese mice harvest energy more efficiently and develop larger body fat stores than their control mates inoculated with gut flora from genetically lean mice.23 Taken together, these animal studies strongly suggest a role for gut microbes in the development of obesity.
This evidence is supported by human studies showing a relative excess of Firmicutes and reduction of Bacteroidetes in the gut flora of obese persons than those from lean persons. In addition, reduction of weight in the former group was associated with partial restoration of gut bacterial composition to the lean pattern. However, the relationship of gut microbes with obesity and NAFLD is complex. Many aspects of this relationship remain unexplored—for instance, how do relatively small differences in harvesting of energy induced by variations in gut microbes translate into clinical obesity, whether the microbes can sense changes in body weight and tailor the efficiency of their energy harvesting process, and whether the changes in gut flora represent a cause or effect of increased body weight. Further, the gut flora show marked inter-individual variability, and the relationship of gut flora with obesity is far from perfect. Thus, although the current human data do show an association between profile of gut microflora and obesity, these do not prove causation. Data from animals are much more convincing; however, these may be species specific and may not apply to humans.

**Gut microbes and insulin resistance.** Insulin resistance plays a central role in the development of hepatic steatosis and also contributes to hepatic inflammation. Several lines of evidence strongly suggest a role for gut microflora in the induction of insulin resistance.

Obese individuals have a higher prevalence of intestinal bacterial overgrowth. Cell walls of gram-negative bacteria contain lipopolysaccharide (LPS) or endotoxin, which can activate an inflammatory cascade through both toll-like receptor (TLR) 4-dependent and TLR4-independent pathways, resulting in upregulation of genes for several cytokines (tumor necrosis factor-α [TNF-α] and interleukin [IL]-6), inducible nitric oxide synthase, nuclear factor-xB (NF-xB), inhibitor of NF-xB, etc. These inflammatory mediators are known to induce a state of insulin resistance. Persons with NAFLD have an increased intestinal permeability than controls. Serum endotoxin levels are higher in patients with type II diabetes mellitus, a classical state of insulin resistance. Modification of gut microbes in mice using probiotics or anti-TNF-α antibodies has been shown to reduce serum inflammatory cytokines levels, improve insulin resistance, and reduce hepatic steatosis at histology. However, extrapolation of these data to humans may be difficult because of differences in diet, genetics, metabolism, environmental factors, and presence of associated disease conditions.

**Progression from NAFLD to NASH.** The classical two-hit hypothesis for NASH considers hepatic steatosis as the first hit that sensitizes hepatocytes to a variety of other insults; one of these insults (the second hit) then induces progression of some cases from NAFLD to NASH (Fig. 1). The proposed second hits have...
included genetic factors, oxidative stress, TLR-mediated signaling in Kupffer cells, and adipose tissue-derived inflammatory cytokines. Gut microbiota may provide another second hit, either through innate immune mechanisms or through excessive endogenous production of alcohol.

Liver is rich in cells of the innate immune system. These cells can recognize exogenous molecules that carry specific pathogen-associated molecular patterns and endogenous substances with danger-associated molecular patterns through their pattern-recognition receptors, including TLRs and nucleotide-bonding oligomerization domain-containing protein-like receptors. Interaction of these receptors with bacterial products leads to activation of several inflammatory pathways, including the inflammasomes. The latter, in turn, activate caspase-1, which cleaves pro-IL-1β and pro-IL-18 into pro-inflammatory cytokines.

Inflammasomes appear to sense and regulate colonic microbiota. Their deficiency in mouse colonocytes is associated with a pathogenic colonic microbial pattern, that is an increase in Bacteroidetes and reduction in Firmicutes. Knockout mice that genetically lack components of inflammasome show pathogenic changes in gut microbiota as well as increased levels of LPS and bacterial DNA (which bind to TLR4 and TLR9, respectively) in portal blood, enhanced hepatic expression of TNF-α, and increased hepatic steatosis and inflammation. In a recent mouse study, TLR4 on Kupffer cells were shown to play a key role in mediating progression from hepatic steatosis to NASH; in contrast, TLR4 deficiency has been shown to attenuate NASH. Excess of pro-inflammatory cytokines, particularly TNF-α, appears to contribute to disease progression in human NASH, too. These findings suggest that a genetic impairment of inflammasome function in some individuals may lead to changes in gut microbiota, which, by increasing the level of liver pro-inflammatory cytokines, may promote progression of NAFLD to NASH.

Human body produces a small amount of alcohol under physiological conditions. Reduction in breath ethanol concentration following neomycin treatment indicates that gut microbiota is the major source of this endogenous alcohol. Endogenous alcohol is efficiently oxidized in the liver by alcohol dehydrogenase. A recent study showed that patients with NASH had an excess of alcohol-producing *Escherichia coli* in their gut and significantly elevated serum ethanol levels. In another study, NASH livers showed a markedly increased expression of ethanol-metabolizing enzymes. Ethanol is also known to increase gut mucosal permeability and serum endotoxin levels, particularly in patients with ALD. These findings, primarily from animal studies, suggest a role for gut microbiota in liver injury of NASH. An altered gut microbiome in persons with NASH may result in increased intestinal ethanol production; this, combined with consequent increased gut permeability, may lead to an increased exposure of liver to ethanol and its toxic metabolites, reactive oxygen species, and bacterial endotoxin, all of which may together promote liver inflammation. Whether this applies to humans needs further work.

**ALD and gut flora.** Liver injury in ALD is characterized microscopically by hepatic steatosis, necroinflammation, and fibrosis. Gut microbes may contribute either directly or indirectly to each of these three components.

The mechanisms of hepatic steatosis and their interaction with gut flora in ALD are likely to be similar to those discussed above in relation to NAFLD.

Hepatic necroinflammation in ALD is the cumulative result of several injurious processes, which include: direct toxic effect of ethanol and its metabolites on the liver; bacterial translocation and endotoxemia secondary to impaired intestinal epithelial barrier; and immune mechanisms directed against protein adducts of metabolites of ethanol and lipids. Several observations support an additional causative role of gut microbes in the pathogenesis of ALD.

Chronic alcohol consumption is associated with colonic dysbiosis, with an increase of Proteobacteria in preference to Bacteroidetes, and endotoxemia. Proteobacteria are gram-negative bacteria that carry LPS in their outer membrane and have been implicated in inducing intestinal mucosal inflammation. Alcohol consumption also results in intestinal bacterial overgrowth, secondary to impared bile flow, intestinal dysmotility, reduced gastric acidity, and altered intestinal immune response. As alluded to above, alcohol also increases gut permeability in a dose-dependent manner, possibly through the action of its metabolite acetaldehyde. Colonic mucosal cells and microbes have limited capacity to oxidize acetaldehyde to acetate. The resultant accumulation of acetaldehyde in colonic mucosal cells disrupts intercellular junctions, increasing paracellular permeability for gut-derived toxins. Another possible mechanism for ethanol-induced gut leakiness is nitric oxide overproduction through activation of transcription factor Snail.

In addition to this transient alcohol-induced increase in permeability, patients with alcohol-related cirrhosis have a persistent increase in gut permeability. Chronic alcohol intake can thus, through a combination of altered bacterial composition and increased permeability, be expected to increase translocation of LPS and other inflammatory products to the liver. LPS engages and activates TLR4 on Kupffer cells in the liver, resulting in production of various pro-inflammatory factors such as cytokines, chemokines, and reactive oxygen species, thus propagating alcohol-induced liver injury.

**Hepatic fibrogenesis.** Fibrosis, i.e. deposition and accumulation of collagen and other extracellular matrix proteins in intercellular spaces, is a healing response that is shared between several forms of liver injury irrespective of the cause, including viral infections, metabolic diseases, toxin exposure, etc. When severe, it results in liver cirrhosis and its attendant serious complications such as portal hypertension, variceal bleeding, ascites, HE, and HCC. Hepatic fibrosis is mediated primarily by activated hepatic stellate cells, along with portal fibroblasts and myofibroblasts; several cytokines, chemokines, immune factors, and LPS are known to activate these cells and hence increase hepatic fibrosis.

Patients with liver fibrosis and cirrhosis have increased number and an altered composition of bacteria in their gut; these changes are more marked in patients with advanced liver disease, as evidenced by higher prevalence of bacterial overgrowth in patients in Child–Pugh class B/C than those in class A. Further, they have increased intestinal permeability and bacterial translocation, caused in part by portal hypertension and vascular congestion.
Culture-independent techniques targeting the hypervariable 3 region of the bacterial 16S rRNA gene have shown a reduced microbial diversity and reduction in Bacteroidetes, and an increase in Proteobacteria and Fusobacteria in patients with liver cirrhosis.\(^{32}\) Although the exact reason for these changes remains unclear, reduced intestinal motility, decreased gastric acidity and pancreatico-biliary secretions, and portal hypertensive enteropathy may all contribute. In an experimental mouse model of liver fibrosis, expression of profibrogenic genes (including transforming growth factor-β, matrix metalloprotease-2, procollagen α1, and tissue inhibitor of metalloprotease-1), serum levels of pro-inflammatory cytokines (TNF-α and IL-6) and bacterial translocation showed progressive increase with increasing fibrosis.\(^{53}\)

Thus, the available data suggest a possible role for altered gut microbiota in liver fibrogenesis. However, majority of data that suggest a pathogenetic relationship are based on animal studies. Human data on the association are limited to observational studies showing qualitative and quantitative alterations in gut microbiota in cirrhosis and are currently inadequate to prove a cause–effect relationship.

**Relationship of gut flora with complications of liver disease**

The clinical course of liver cirrhosis is frequently complicated by development of GI bleed, HE, renal failure, or spontaneous bacterial peritonitis (SBP), leading to a detrimental effect on liver function and poorer clinical outcomes. Altered gut microbiome may also influence the risk of development and outcome of these complications.

**SBP and other bacterial infections.** Patients with cirrhosis have an increased risk of hospital-acquired infection than non-cirrhotic controls. Common bacterial infections in patients with liver cirrhosis include SBP, respiratory tract infections, urinary tract infection, and generalized sepsis. A large majority of these infections are caused by gram-negative enteric bacilli, suggesting an origin in the gut.

Patients with chronic liver disease have an overgrowth and translocation of gut bacteria, as evidenced by an increased presence of bacterial DNA\(^{54}\) as well as antibodies against microbes\(^{55}\) in their circulation. Intestinal permeability is increased in cirrhotics with ascites, history of SBP, and higher Child–Pugh score.\(^{46}\) In one study, the bacteria showing translocation from bowel to mesenteric lymph nodes belonged mostly to Enterobacteriaceae family, *Enterococcus* group and some *Streptococcus* species,\(^{57}\) that is similar to those causing infections in patients with cirrhosis.\(^{38}\)

Cirrhosis in rats has been shown to cause a shift in gut bacterial flora toward more hydrophobic flora with better adherence to the mucosa, facilitating translocation across the mucosa.\(^{39}\) Stool culture studies in patients with cirrhosis show an overgrowth of pathogenic *E. coli* and *Staphylococcus* species.\(^{60}\) Studies on ascitic fluid, portal blood, mesenteric lymph nodes, and ileal contents have shown that bacterial translocation was more frequent in rats with ascites and SBP than in rats with ascites but no SBP or in healthy rats\(^{46}\); the bacterial species isolated in mesenteric nodes or ascites were similar to those in the rat intestine, suggesting translocation of bacteria from gut as the source of SBP\(^{41}\) Further, cirrhosis is associated with numerous defects in immune response, including impaired leukocyte function,\(^{63,64}\) low ascitic fluid levels of components of the complement system,\(^{65}\) reduced phagocytic activity of reticuloendothelial cells,\(^{66}\) reduced antibody-mediated and complement-dependent bacterial killing,\(^{67}\) and reduced proliferation and γ-interferon synthesis by intraepithelial lymphocytes\(^{68}\); these could contribute through reduced clearance of translocated bacteria.

**HE.** HE encompasses a wide spectrum of abnormalities, ranging from subclinical alterations in neuropsychiatric tests (minimal HE) to overt neuropsychiatric manifestations of varying severity (clinical HE grade I–IV) in patients with acute or chronic liver failure. Pathogenesis of HE is poorly understood and appears to be multifactorial; several pieces of evidence support a role for gut microbes in this process.\(^{69}\)

Intraperitoneal administration of LPS in a mouse model of cirrhosis is associated with induction of pre-coma and worsening of cytotoxic brain edema.\(^{70}\) Bacterial overgrowth is more common in patients with liver cirrhosis and minimal HE than in those without the latter.\(^{37}\) Liu *et al.* reported an overgrowth of pathogenic *E. coli* and *Staphylococcus* species in patients with cirrhosis and minimal HE.\(^{60}\) Administration of probiotics and fermentable fiber resulted in an increase of non-urease-producing *Lactobacilli* over pathogenic bacteria with a significant reduction in blood levels of ammonia and endotoxin, reversal of minimal HE, and improvement of Child–Pugh score. A meta-analysis of the effect of probiotics, prebiotics, or symbiotics, which modulate gut flora, has shown significant improvement in minimal HE.\(^{71}\)

Increased blood ammonia, leading to astrocyte swelling followed by brain edema, is believed to play a central role in the pathogenesis of HE. Ammonia is produced from catabolism of glutamine in the small bowel and that of undigested proteins and urea in the colon. Several other products of bacterial metabolism also have neurotoxic effects, and their levels are increased in persons with cirrhosis; these include phenols produced from aromatic amino acids such as phenylalanine mercaptans produced from sulfur-containing amino acids such as methionine, and short- and medium-chain fatty acids. Gut bacteria also produce endogenous benzodiazepine-like compounds, which impair astrocyte function and gamma-aminobutyric acid-ergic neurotransmission; blood levels of these compounds are increased in patients with liver cirrhosis, with most marked elevations in patients with advanced disease.

In addition, infections are known to be a major precipitant for HE in patients with liver cirrhosis, and, as discussed in the previous section, gut microbes are the most important source of such infections.

**HCC.** HCC, a common complication of liver cirrhosis, is believed to result from long-standing liver inflammation, with ongoing cell death and regeneration. As already discussed, gut microbes and their products such as LPS mediate hepatic inflammation through TLR4 receptor. TLR4 activation is also believed to influence proliferation, resistance to apoptosis, and propensity of tumor cells to invade tissue and metastasize.\(^{72}\) Reduction of endotoxin level through administration of an antibiotic or ablation of its receptor TLR4 has been shown to prevent tumor growth in mice.\(^{73}\)
In another study, genetic TLR4 inactivation, gut sterilization, or GF status decreased the development of HCC by around 80%, whereas prolonged administration of low-dose LPS increased HCC development.74 Other pathways that mediate inflammation such as NF-κB and c-Jun N-terminus kinases have also been linked with carcinogenesis,75,76 although the data on those are less extensive.

Overall, the current evidence favoring a role for gut microbes in the pathogenesis of HCC is quite limited, and further data, particularly those from humans, are necessary.

**Therapeutic role of gut microbiota alteration in liver disease**

As indicated above, gut microbes appear to play a pathogenetic role in causation of several forms of liver disease and their complications. Hence, it is plausible that manipulation of gut microflora may favorably influence the course and outcome of liver disease. This may be done using prebiotics, probiotics, non-absorbable antimicrobial agents such as rifaximin, non-absorbable disaccharides such as lactulose or lactitol, or fecal transplantation. In fact, these agents have been tried, either alone or in various combinations, in several clinical situations related to liver diseases, such as treatment of NAFLD, prevention and treatment of overt or minimal HE, and prophylaxis of SBP, often with beneficial results.

A better understanding of perturbations in gut flora using the newly developed tools should allow us to refine these treatments and improve their efficacy in the next few years. It is possible that treatment of liver disease in the near future would be personalized based on the study of gut flora in an individual patient.

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