Microbiome and NAFLD: potential influence of aerobic fitness and lifestyle modification

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Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease that encompasses a spectrum of liver pathologies ranging from simple steatosis to more severe nonalcoholic steatohepatitis (NASH), which includes the development of inflammation and fibrosis. Alarmingly, NAFLD is considered the most common chronic liver disease in the United States, currently estimated to affect 80 million–100 million Americans (107, 149, 180). NASH poses major health risks for comorbidities including insulin resistance (30), cardiovascular disease (119, 190), and increased mortality (62). NAFLD development is often linked to reduced hepatic mitochondrial function, increased de novo lipogenesis, and enhanced free fatty acid uptake (88); however, the underlying mechanisms triggering the transition to the more severe phenotype of NASH are not fully understood.

Lifestyle changes such as diet and exercise and elevated aerobic fitness are related to improvements in metabolic diseases, including NAFLD/NASH. These beneficial effects may be elicited through changes in the gut microbiome, a resident of over 2,000 distinct species including bacteria, archaea, fungi, and viruses; a collection that represents 150 times more genes than the human genome (29). Unhealthy lifestyles or physiological status, such as obesity, pose a dysbiotic microbial signature (98, 99), particularly by having greater capacity to metabolize carbohydrates and provide energy to the host (176). Changes in microbial composition are also implicated in steatosis (7, 154, 159), NASH (69), and fibrosis (15), with associations between gut microbial products and NAFLD (178), a concept referred to as the gut-liver hypothesis.

Understanding the interaction between changes in the microbiome composition/metabolite production with hepatic metabolism is crucial in understanding the gut-liver axis and potentially NAFLD progression. In fact, obese patients with liver fibrosis exhibit elevated blood microbiota compared with patients with no fibrosis (94), suggesting that microbiota trans-
locating from the gut to the portal circulation may be a stimulus contributing to NASH severity. The metabolic capacity of the microbiome to produce metabolites in response to environmental factors (i.e., diet, exercise, aerobic fitness etc.) also may have a direct effect on the liver and systemic metabolism; however, the mechanisms explaining this relationship are still not well understood. The overall objectives of this review are to 1) define the significance of the microbiome and the gut microbiome-liver hypothesis, 2) discuss the microbial products that may significantly impact hepatic metabolism, and 3) provide evidence of how different lifestyle modifications including diet, exercise, and aerobic fitness change the microbiome, with discussion on the implications to NAFLD/NASH.

Microbiome Differences between Preclinical Models and Humans

Throughout this review we will reference rodent, pig, and human microbiome studies and therefore deem it necessary to discuss the compositional and functional similarities. The use of preclinical models in gut microbiota research has been reviewed previously (130). There are substantial microbial gene differences, as well as compositional differences at the genus and species taxonomic levels between mice and humans; however, the functions of those genes and phylum taxonomic composition appear to be similar (197). Rats arguably may be more representative of the human gut microbiota, as they take on a similar microbial composition to humanized donors (130, 195). In addition, rodent models have the greatest microbial density within the cecum, while humans have high microbial composition and diversity throughout the large bowel. In miniature swine, obesity-related changes in gut microbial composition are similar to what has been found in humans (137), and overall, swine have similarities in gastrointestinal anatomy, physiology, and immunology to what is seen in humans (104). Thus, the gut microbial metabolites discussed here are produced by both humans and preclinical models and have similar metabolic consequences in reference to NAFLD and NASH, with the exception of exact bile acid species in rodent models that we will discuss below.

Bile acids. Bile salts are synthesized in the liver and secreted into the duodenum to act as detergents in aiding in the solubilization, digestion, and absorption of lipids and lipid-soluble vitamins throughout the large intestine (147). The main primary bile acids produced in humans are chenodeoxycholic acid (CDCA) and cholic acid (CA), while mice produce primarily CA and muricholic acids (MCA, predominantly beta-MCA) (184). A majority of primary bile acids get reabsorbed by high-affinity active transport in the ileum and are transported back into portal circulation; however, 5% of bile acids reach the large bowel where they can be 7α-β-dehydroxylated into the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) (148). Recent evidence suggests that bile acid metabolism by the gut microbiome through bioconversion (i.e., deconjugation, dehydrogenation, and dehydroxylation) is directly related to NAFLD and the progression to NASH (148, 153). Specifically, binding of primary or secondary bile acids to bile acid receptors farnesoid X receptor (FXR) and G protein-coupled bile acid receptor (TGR5) promotes a signaling cascade that can influence hepatic lipid, glucose, and cholesterol metabolism. Both FXR and TGR5 activation lowers hepatic inflammation and steatosis (101), and dysregulation of lipid homeostasis has been linked to lower hepatic fibroblast growth factor 19 (FGF19) in NAFLD (155). Furthermore, a recent multicenter randomized, placebo-control trial assessing the efficacy of obeticholic acid, a potent activator of FXR, in noncirrhotic NASH patients found improvements in liver histology; however, the long-term effects of this treatment needs further evaluation (128). Changes in bile acid species (e.g., primary vs. secondary bile acids) from gut microbiome metabolism as well as changes in enterohepatic flux could impact the binding of bile acids to these receptors and affect NAFLD risk.

In humans, the inability to 7α-hydroxylate secondary bile acids returning to the liver causes accumulation in the bile acid pool (147). Together, the microbiota, intestinal transit time, and diet regulate bile acid pool composition and size. Indeed, animal-based diets high in saturated fat shift the microbiome to increase more bile-tolerant genera (i.e., Alistipes, Bilophila, and Bacteroides) and bile acid conjugation to taurine increases DCA levels, which has been shown to promote intestinal inflammation (49, 124) and perhaps hepatic inflammation. Interestingly, advanced cirrhosis patients exhibit decreased conversion of primary to secondary bile acids, increased fecal pathogenic Enterobacteriaceae, and decreased 7α-dehydroxy-lating microbes Blautia and Lachnospiraceae (80). Moreover, circulating bile acids have been shown to be elevated with NASH (57, 81), and transcriptomic analysis of livers from NASH patients reveals downregulated bile acid metabolism (89). Furthermore, a recent study in NASH patients found increased total fecal bile acids, CA and CDCA compared with healthy controls, which correlated with a decreased relative abundance of Bacteroidetes and Clostridium leptum, a species that can 7α-dehydroxylate and deconjugate bile acids (123). This is also seen in non-obese patients with NAFLD, where there is a decrease in microbial diversity and Firmicutes involved in 7α-dehydroxylation (187).

To summarize, increasing the amount and type of bile acids (taurine vs. glycine-conjugated bile acids) entering into the large bowel can increase Firmicutes involved in 7α-dehydroxylation primary bile acids into toxic secondary species. The change in bile acid delivery into the large bowel can promote the growth of pathogenic/lipopolysaccharide (LPS)-containing bacteria like Enterobacteriaceae (146). Overall, understanding this complex interaction between enterohepatic flux, the microbiome, and liver metabolism is vital and may be a viable target in combating NAFLD/NASH.

Short-chain fatty acids. Short-chain fatty acids (SCFA) are the fermentative end products of undigested carbohydrate/dietary fiber entering the large bowel. The three primary SCFA are acetate, propionate, and butyrate, and under normal physiologic conditions, they are produced in a molar concentration of 60:20:20, respectively, in the colon and feces (13, 37). The impact of SCFA production on liver and whole body metabolism may be viewed as either beneficial or detrimental depending on physiological status. Under normal physiological conditions, SCFA production has positive effects on host gut health by lowering intestinal pH. In addition, butyrate is known to reduce the growth and motility of colonocyte cancer cell lines by inducing apoptosis (132), is the primary energy source of normal colonocytes (151), and promotes gut epithelial
integrity by increasing tight junction assembly (138). Furthermore, fermentable fiber intake and SCFA have been found to be beneficial in fighting obesity, in part through increasing circulating anorexic gut hormones peptide YY and glucagon-like peptide 1 (GLP-1) to promote satiety (25, 59, 131). Specifically in the liver, dietary fiber and direct SCFA administration at physiologically relevant doses reduces hepatocellular triglyceride concentrations, liver cholesterol synthesis, and de novo lipogenesis in rodents (11, 68, 79) and humans (5, 95, 166).

Although potentially beneficial under normal physiological conditions, the gut microbiome in an obese and NAFLD state has been linked to overproduction of SCFA in the proximal large bowel. In fact, SCFA content is greater in fecal content of obese humans as well as showing a concomitant decrease in gross fecal energy content compared with lean individuals (6, 156), highlighting potentially increased energy extraction from the diet in obese individuals. In addition, direct SCFA infusion at high physiological concentrations in the colon of humans and cecum of mice over a course of 30 min and 6 h, respectively, drives up hepatic de novo lipogenesis, cholesterol synthesis, and glucose production (47, 194). Specifically, acetate and butyrate can be used as substrates for de novo lipogenesis and cholesterol synthesis, while propionate is a precursor for gluconeogenesis and cholesterol synthesis (47). These constant infusions over time yield SCFA levels well above what is observed to be beneficial in other reports. Moreover, high-fat diet (HFD)-induced obesity in mice and humans is marked with an increase in Firmicutes and decrease in Bacteroidetes and a greater propensity for energy harvest in the form of SCFA from the diet and contributes to increased energy harvest for the host (84, 97, 176). On the other hand, germ-free mice with very low SCFA production are protected against diet-induced obesity (7). Additionally, we have recently found that intrinsically low aerobically fit rats on a low-fat diet have a greater abundance of SCFA-producing microbes and an increased predicted metabolic potential for energy and carbohydrate metabolism compared with their highly aerobically fit counterparts, which likely contributes to elevated NAFLD susceptibility in this model (134). Collectively, whether SCFA production is beneficial vs. detrimental on hepatic metabolism may be attributed to the concentrations of SCFA administered or produced by dietary fiber fermentation, as well as administration site (i.e., oral, cecal, or rectal delivery). Additional studies in this area are definitely warranted.

The role of SCFA acting as signaling molecules through their G protein-coupled receptors (GPCRs) has gained considerable attention in recent years with regard to inflammation and metabolism (85). Gut microbial-derived metabolites, including SCFA, activate GPCRs similarly to neurotransmitters and hormones [see Husted et al. for excellent review (76)]. Free fatty acid receptor (FFAR) 2 and 3 have affinities for all SCFA; however, they are primary targets of acetate and propionate, while butyrate preferentially targets FFAR3 and hydroxybutyric acid receptor 2 (HCA2). In a lean healthy state, it is likely that SCFA serve as gatekeepers in the gut by maintaining or recruiting leukocytes into the lamina propria to promote an appropriate immune response from microbial-initiated insults (76). However, in a chronic state of obesity and low-grade inflammation where gut microbial dysbiosis is apparent, microbial products such as LPS and pathogen-associated molecular patterns (PAMPs) compromise epithelial function, increase leukocyte recruitment to the gut, and propagate an escalated inflammatory response (76). It is proposed that there is an interplay between GPCRs and toll-like receptor (TLR) signaling on the innate immune response through their synergistic effect on the release of IL-17F (67, 76). Both of these signal cascades promote NF-kB through MAPK activation, stimulating inflammation (76). Together, knowing whether in a state of obesity that SCFA-GPCRs and TLR-4 signaling is synergistic or antagonistic is intriguing and may be a vital piece in the gut-liver hypothesis as it relates to NAFLD/NASH.

The expression and function of GPCRs and activation with SCFA in the liver are still largely unknown (76). In particular, the evidence of FFAR2 manipulation on changing hepatic steatosis and energy metabolism is conflicting. Initially, FFAR2-deficient mice were reported to have lower body fat, increased lean mass, and lower liver weight and liver triglycerides compared with wild-type mice (14). However, more recent evidence shows HFD-fed FFAR2-deficient mice have greater weight gain, adiposity, and insulin resistance compared with wild-type mice (86), whereas overexpression of FFAR2 in adipose tissue elicited protective effects from diet-induced obesity and hepatic steatosis (86). Furthermore, FFAR2-deficient mice in a germ-free environment are protected from HFD-induced increases in obesity outcomes, demonstrating a potential link between the interaction of the microbiome, SCFA-FFAR2 binding, and energy metabolism (9, 86). As stated previously, FFAR2 could also have direct effects on gut inflammation, increase gut permeability, and cause a greater propensity for microbial products to reach the liver and contribute to NAFLD (113). However, more definitive studies are needed to better establish the connection between the gut microbiota, GPCRs, and NAFLD risk.

The contradictory observations revealing the potential benefits vs. detriments of SCFA production on hepatic metabolism highlight the complexity of their roles in the gut-liver hypothesis. The microbiome changes in the pathological state of obesity and NAFLD suggest that overproduction of SCFA may contribute to hepatic steatosis by being a significant source of energy for the host; however, fermentation of certain dietary fibers yields SCFA at less metabolically taxing concentrations and therefore elicits benefits to host metabolism potentially through their actions on GPCRs and maintenance of epithelial immune homeostasis. Overall, more mechanistic insight and clarity are needed in elucidating the interaction of the gut microbiota, SCFA production, and the potential contribution to NAFLD development and progression.

**LPS/endotoxemia and intestinal permeability.** Obesity is characterized by a state of chronic low-grade systemic inflammation caused by increased circulating proinflammatory cytokines, as well as LPS, a major component of the outer membrane of gram-negative bacteria (141). Prolonged Western style diet feeding causes increases in gram-negative microbiota in the gut promoting increased intestinal permeability and greater LPS/endotoxin delivery into portal and systemic circulation, a condition known as metabolic endotoxemia (22, 23). In the small intestine, LPS can also incorporate into the mixed micelle from its attachment to dietary fat and then be absorbed and enter the general circulation (52). Endotoxemia has been witnessed in both humans and genetically obese and HFD-induced rodent models (22, 23, 60, 70, 158, 170, 179). Inter-
that are initiating this response. However, more mechanistic experiments are needed to deter-
mine whether it is LPS alone or other inflammatory mediators as an inflammatory stimulus in the progression of NAFLD.

Interestingly, NAFLD patients exhibit increased indexes of small intestinal bacterial overgrowth (SIBO) with a concomi-
tant reduction in duodenal mucosal crypt and villus ZO-1 (117). Similarly, HFD-fed mice have increased gut permeabil-
ity and decreased expression of intestinal tight junction proteins (23). These data suggest that obesity-induced increases in circulating LPS/endotoxin are driven by gut microbiota inter-
actions with the diet and with the intestinal epithelium.

LPS elicits a signaling cascade known to promote NASH (33, 72, 160, 196). Specifically, LPS binds to the hepatic TLR-4/MD-2/CD-14 complex, triggering an inflammatory re-
sponse mediated through myeloid differentiation primary re-
response gene 8 (MyD88)-dependent and -independent path-
ways (i.e., increased TNF-α, NF-κB, and IL-6 among others). Interestingly, NAFLD patients exhibit increased TLR-4 ex-
pression in hepatic progenitor cells, bile ducts, and interlobular bile ducts (181), and hepatic inflammatory and fibrogenic signals appear to be TLR-4-mediated in Kupffer cells and hepatic stellate cells (150). Early studies from Cani et al. (22) found that CD-14-deficient mice were protected against diet-
induced obesity, suggesting that some of the mechanistic features of obesity involve the actions of LPS signaling. Furthermore, TLR-4 mutant mice are resistant to the deleteri-
ous hepatic NASH phenotype caused by a methionine/choline-
deficient diet (150). High-saturated-fat diet (163), and genetic obesity (ob/ob) mice (164). Overall, these data suggest that the combination of increased gram negative bacteria and gut perme-
ability elevate the hepatic exposure to LPS, which may act as an inflammatory stimulus in the progression of NAFLD. However, more mechanistic experiments are needed to determine whether it is LPS alone or other inflammatory mediators that are initiating this response.

**Bacterial DNA.** In addition to LPS, other gut-derived prod-
ucts are implicated in obesity and NAFLD/NASH. Bacterial DNA are constituents of the gut with unmethylated cytosine-
guanosine dinucleotides (163), where upon translocation into portal circulation can act through TLR-9 to initiate a hepatic inflammatory response by acting on immune cells (163). In mice, hepatic stellate cells from TLR-9 knockout mice are protected against fibrogenic stress (61), and TLR-9 knockout mice are largely protected against choline deficient and methi-
onine/choline deficient diet-induced NASH (72, 120). These studies collectively demonstrate that bacterial DNA signaling through TLR-9 likely plays a role in the development and progression of NAFLD. Frances et al. (58) first showed that bacterial DNA was implicated in the clinical outcomes of NAFLD, as advanced cirrhosis patients had increased levels of bacterial DNA in the blood and ascitic fluid. Furthermore, this continued after therapeutic paracentesis (removal of ascitic fluid from abdomen) (58), suggesting that translocation of bacterial DNA was ongoing. Another group found that obese patients with fibrosis have increased bacterial DNA, a finding that occurred in the absence of differences in other metabolic markers such as serum cholesterol, C-reactive protein (CRP), glutamic-pyruvic transaminase (GPT), or gamma-glutamyl transpeptidase (GGT) (94). These changes occurred concur-
tently with differences in the blood and fecal microbiome, suggesting that bacterial products are able to translocate from the gut to the circulation (94). While the involvement of bacterial DNA in the pathogenesis of NAFLD and NASH has been reported in humans, the direct mechanisms of action are understudied and future investigations are warranted.

**Gut microbial components and inflammasome activation.** Inflammasome activation plays a critical role in the micro-
bioe-liver axis and has been reviewed previously (10). Inflammasomes are a collection of large multiprotein complexes including NOD-like receptors (NLR) NLRP3, NLRC4, absent in melanoma-2, and NLRP6 that upon recognition of microbial signals promote an inflammatory response (10). Indeed, both LPS and bacterial DNA activate TLR-dependent mechanisms, causing an inflammatory cascade; however, a range of bacterial components and metabolites may initiate the inflammatory response in an inflammasome-dependent manner. Both NLRP3 and NLRP6 inflammasomes, as well as the effector protein IL-18, negatively regulate progression of NAFLD to NASH through changes in the microbiome (72). Specifically, inflam-
masome-deficient mice exhibit elevated steatosis and NASH, which is transmissible to wild-type mice upon cohousing (72). The exacerbated steatosis and inflammation were linked to increased gut pathogen-associated molecular patterns (PAMPs) that act as agonists for hepatic TLR4 and 9, inducing TNF-α expression and promoting NASH. Inflammasome-deficient mice in response to high-fat and methionine- and choline-
deficient diets also exhibit an expansion of Porphyromon-
adaeae, a taxon linked to chronic liver disease (8, 72). Other studies have linked *Porphyromonas*, a genus within Porphyromonadaeae, to metabolic diseases such as atherosclerosis and diabetes mellitus (8, 109); however, further work is needed to demonstrate the role of this taxon in the onset and patho-
genesis of NAFLD/NASH. Overall, the combination of NLRs and TLRs shape the metabolic progression of NAFLD and NASH. Activation of these receptors seems to be driven by modulation of the microbiome, potentially through PAMP activation of inflammatory pathways.

**Dietary Causes of Dysbiosis**

**High fructose.** Increases in food consumption in the US over the past three decades are due largely to increased carbohydrate intake (28), and excessive carbohydrate consumption is linked to NAFLD (127). Fructose consumption, with the introduction of high-fructose corn syrups, has more than doubled in the last three decades (102). Perhaps most disturbing is that fructose-
and sugar-sweetened beverages account for ~15% of consumed calories in US adolescents (188) or ~75 g of fructose per day. It also is estimated that 20% of teenagers consume ≥25% of

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their daily calories from fructose (17). Consumption of fructose-containing beverages, either as fructose or sucrose, contributes more to the development of NAFLD than do isocaloric alternatives (127). In addition, Stanhope et al. (162) demonstrated that equal consumption of fructose-sweetened beverages, but not glucose-sweetened beverages, induces dyslipidemia and insulin resistance. Moreover, high fructose consumption dramatically elevates hepatic de novo lipogenesis (183), a major pathway in hepatic steatosis development in NAFLD patients (50).

Dietary fructose is also linked to NASH onset (183) and fibrosis/NASH severity (1). High dietary fructose is associated with dysbiosis in the proximal colon causing microbes from the large bowel to enter the distal ileum, a condition known as SIBO. SIBO causes increased gut permeability (12, 102, 183), promoting long-term low grade endotoxemia (199), and can cause LPS-dependent activation of hepatic Kupffer cells (161) and stellate cells (157). Indeed, only 25–50 g of fructose can be absorbed by the small intestine per day (143, 174), which is well below the level that many adults and children consume. Both SIBO and low-grade endotoxemia have been reported in patients and animals with NAFLD and NASH (26, 108, 117, 140, 168, 192) and are positively linked to increased fructose intake (170). It is not entirely clear whether fructose-induced dysbiosis and intestinal permeability are the primary mediators in NASH development and progression. Interestingly, calorically controlled fructose consumption in nonhuman primates caused signs of endotoxemia and liver injury without apparent changes in the microbiome (83), suggesting that fructose-induced gut dysbiosis may only happen in the presence of excess energy intake and may cause liver injury independently of the microbiome. However, targeting the gut microbiome to reduce liver inflammation and improve intestinal barrier function has shown promise in rodents (23, 53, 77), making it a potential therapeutic target in fructose-induced NAFLD/NASH in humans. Overall, the effects of high fructose consumption on the human microbiome in relation to NAFLD/NASH is relatively underdeveloped and future investigations are warranted.

High-fat/Western diet. It is well established [see reviews (73, 96)] that high-fat/Western style diet feeding causes microbial dysbiosis that is linked to NAFLD. Specifically, diet-induced microbial dysbiosis can alter gut motility and increase gut inflammation, which may initiate the microbiota’s influence on the liver (96). In general, HFD-induced dysbiosis is defined as a decrease in species richness/α-diversity and robust changes in microbial composition, including decreased Bacteroidetes and increased Firmicutes and Proteobacteria. Changes in these microbial phyla have been associated with negative health outcomes, including increased energy harvest from the diet, as well as increased systemic inflammation by enhancing gut permeability. To this end, understanding the mechanisms of how high fat/Western diet-induced microbial dysbiosis acts as a stimulus to the onset of NAFLD/NASH is pertinent.

Swine have similar metabolic features, cardiovascular systems, proportional organ sizes, and the structure of their gastrointestinal tract to humans (36, 104, 186). In addition, adult Ossabaw pigs develop obesity and the metabolic syndrome when fed an HFD (16, 51, 126) and a severe NASH phenotype when fed a Western diet high in fat, fructose, and cholesterol (92). We have recently shown that juvenile Ossabaw swine fed a Western diet from postweaning develop obesity, hyperlipidemia, adipocyte hypertrophy, and systemic insulin resistance (133, 172, 182). In terms of microbiota composition, HFD-induced changes on the microbiome in Ossabaw swine are similar to what is observed in humans (137). Utilizing 16S 454-Pyrosequencing, we have data showing that 95% of the cecal microbiota in pigs is represented by Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria (Fig. 1A), which is also consistent with observations in the human microbiome (38). Western diet feeding to induce obesity also resulted in a sixfold increase in Proteobacteria, and more specifically Gammaproteobacteria at the class level and Enterobacteria at the order level (Fig. 1, A and B). This expansion of Proteobacteria, and in particular Enterobacteriaceae, is an observation consistent in children with NASH (204). Utilizing RNA sequencing, transcriptomic analyses revealed LPS signaling as the top upregulated pathway in the liver of obese vs. lean pigs (Fig. 2). This included the activation of IFN-γ, IL-6, TNF-α and IL-1β, which were upstream of NF-κB and STAT signaling, as well as robust increases in genes involved in hepatic fibrosis and stellate cell activation (data not shown). Collectively, we hypothesize in our Ossabaw swine model of NASH that Western diet-induced obesity causes profound cecal dysbiosis promoting a bloom of LPS-containing Proteobacteria and increased gut inflammation and permeability, which allows LPS entry into the portal circulation and serves as a stimulus for the severe NASH phenotype observed in this model.

**Lifestyle Modifications.**

**Change in diet composition.** Changes in dietary composition are known to influence disease and microbiome composition/function. It has been shown that the microbiome can be altered within 1 day in rodent studies (177), whereas, in humans, this change occurs 3–4 days after a dietary switch and the microbiome will metabolically change to adapt to diet composition (i.e., plant vs. animal protein sources) (42, 185). A comparison of fecal microbiomes of healthy European children primarily on a Western diet and rural African children on a high-fiber low-fat diet revealed European children having greater Firmicutes/Bacteroidetes ratio, suggesting a greater “obesogenic” microbiome (43). Furthermore, European children exhibited greater fecal Proteobacteria, and more specifically known LPS-containing Enterobacteriaceae (i.e., *Escherichia* and *Shigella*), and also increased fecal SCFA (43).

Dietary fat composition also affects the microbiome and its metabolic end products. Devkota et al. (49) demonstrated that dietary saturated vs. polyunsaturated fat changes microbial composition and metabolism, including increases in the sulfite-reducing pathobiont, *Bilophila wadsworthia*. Furthermore, a change to saturated fat promotes hepatic taurine conjugation of bile acids, providing a source of sulfur for microbes like *B. wadsworthia* that promote gut inflammation. Saturated fat consumption is also linked to reduced microbial diversity, increased Firmicutes-to-Bacteroidetes ratio, and increased body weight, plasma insulin, and hepatic triglycerides in mice (45), whereas high-carbohydrate diets have been shown to increase the relative abundance of probiotic *Bifidobacteria* and reduce fasting glucose and cholesterol (56). High glycemic index carbohydrate intake caused a decrease in Bacteroidetes,
whereas low-glycemic-index diets and high saturated fat promoted the butyrate producer *Faecalibacterium prausnitzii*. Furthermore, a strong negative correlation was observed with decreased Bacteroidetes and body weight gain, and increased fecal SCFA concentrations were observed with a saturated fat diet. Overall, this demonstrates that the microbiome is influenced by macronutrient source (i.e., saturated vs. unsaturated fat and complex vs. simple carbohydrate), which can promote dysbiosis and poor physiologic outcomes.

Few studies have investigated the interface of dietary protein, the microbiome, and NAFLD. Bacterial fermentation of branched-chain amino acids valine and leucine yields branched-chain fatty acids (BCFA) isovalerate and isobutyrate, which have been linked to insulin resistance and the metabolic

Fig. 1. Predominant cecal microbial phyla (A) and specific classes of Proteobacteria (B) changed between obese and lean (n = 6 each) 20 wk old Ossabaw swine. All values are presented as mean % relative abundance (A) and means ± SE (B). *Difference between lean and obese (P < 0.05).

Fig. 2. Hepatic RNA sequencing pathway analysis, utilizing Ingenuity Pathway Analysis, of genes impacted by lipopolysaccharide (LPS) signaling in Western diet-fed pigs.
syndrome (129). However, the impact of protein source on BCFA concentrations, the microbiome, and NAFLD is not well understood. More recently, dietary soy vs. milk protein has shown protective effects on Western diet-induced hepatic steatosis potentially linked through the microbiome. In rats, dietary soy protein increased cecal microbial diversity compared with casein or fish meal (4) and increased fecal relative abundance of *Lactobacillus* spp. and reduced *Allobaculum* and *Parabacteroides* compared with a high-cholesterol AIN76 diet substituted with casein (93). In addition, evidence in Western diet-fed golden Syrian hamsters demonstrated that soy vs. milk protein isolate intake elicited increased microbial diversity that was associated with decreases in hepatic cholesterol and triglycerides (19). In addition, our laboratory has recently been interested in studying the gut-liver connection with protein source utilizing the OLETF rat model, an established model of nonalcoholic fatty liver disease (NAFLD) (135, 144, 145). We found that soy protein isolate modulates the fecal microbiome and is linked to decreased hepatic steatosis when compared with an isocaloric diet high in milk protein isolate (135). Specifically, soy protein-fed rats exhibited decreased genera in *Clostridium* cluster XIVa, which has species that can 7α-dehydroxylate primary bile acids to the secondary bile acid, DCA (78, 135, 146, 148). Furthermore, dietary soy protein lowered serum cholesterol, which exhibited an inverse relationship with elevated *Lactobacillus*, a genus known to have bile salt hydrolase activity that can lower serum cholesterol through decreasing cholesterol absorption or increasing bile acid synthesis (44). Interestingly, cholesterol synthesis genes increased with soy protein-feeding, which is consistent with the feedback regulation of the cholesterol synthetic pathway in the presence of cholesterol-lowering agents (135). Hepatic gene expression of bile acid receptor FXR and FGF19 and their downstream targets were also increased with soy intake, suggesting differences in bile acid metabolism may be influencing hepatic lipid metabolism (135). Overall, dietary protein derived from soy vs. milk impacts microbial populations that influence hepatic cholesterol and lipid metabolism. However, further study is needed to elucidate the mechanisms of actions of protein source on the microbiome in relation to NAFLD.

**Pre- and probiotics.** Pre- and probiotic supplementation may be effective at combating NAFLD/NASH. Prebiotics are defined as indigestible food ingredients that stimulate the growth/activity of beneficial gut microbes (173). Dietary probiotics are living bacterial strains that confer benefits to the host (71). With regard to obesity and metabolic syndrome, prebiotics increase gut epithelial tight junction proteins occludens and ZO-1, promote endocrine action of peptide YY and GLP-1 to promote satiety, as well as decrease fat mass and insulin resistance [reviewed in (46)]. Furthermore, dietary oligofructose increases endocrine function to promote satiety with a concomitant decrease in body weight (136). Like prebiotics, probiotics create a competitive advantage for commensal microbes to promote barrier function and GLP-1 secretion to improve glucose tolerance and satiety, which correlates with SCFA production (91, 200).

The clinical significance of pre- and probiotics on the microbiome in relation to host metabolism and combating metabolic syndrome has been studied and previously reviewed (87, 111). In terms of prevention and treatment of NAFLD, randomized clinical trials are lacking in assessing the effective-ness of pre- and probiotics [systemic reviews by (103, 167)]. A study in obese NAFLD patients found VSL#3 probiotic supplementation improved markers of liver function and injury (106). In addition, a symbiotic dietary intervention (i.e., combination of a pre- and probiotic) consisting of *Bifidobacterium longum* and fructo-oligosaccharide for 24 wk in NASH patients reduced TNF-α, CRP, aspartate aminotransferase (AST), homeostatic model assessment-insulin resistance, and LPS, as well as decreased NASH activity index (110). Pre- and probiotics also have been shown to protect rodents from fructose-induced liver damage (18, 160). In particular, *Bifidobacteria* have been shown to reduce LPS and improve intestinal barrier function (65, 152, 189), and *Bifidobacterium lactis* Bb12 supplementation has been shown to reduce Enterobacteriaceae family bacterial contents in preterm infants (121). Although these studies suggest a benefit of prebiotic, probiotic, and symbiotic supplementation to NAFLD, future randomized clinical studies investigating type/strain as well as effective doses are needed to determine their efficacy in combating the disease.

**Weight loss/calorie restriction.** Calorie restriction-induced weight loss is effective in combating NAFLD (27), and a portion of this benefit may be due to changes in the microbiome. Dao et al. (40) characterized microbiome alterations of obese persons entering a calorie reduction program. At baseline, participants with higher levels of *Akkermansia muciniphila*, a mucin-degrading bacteria, had improved indexes of glucose metabolism, serum leptin, and serum AST levels. Interestingly, caloric restriction decreased *A. muciniphila* in subjects with elevated baseline levels and increased it in subjects with lower baseline levels; however, glucose metabolism was improved regardless of changes in this microbial species (40). In a study comparing calorie restriction vs. bariatric surgery, calorie restriction increased Firmicutes and decreased Bacteroidetes, along with an increased capacity for SCFA production, whereas bariatric surgery patients exhibited a decrease in this ratio (39). Interestingly, both interventions decreased body mass and improved blood glucose and triglycerides, suggesting these benefits may be independent of changes in the microbiome. Thaiss et al. (169) found that weight loss due to caloric restriction in mice induces changes in microbiome composition and metagenomic function that promote a greater weight regain after cyclic HFD feeding. After one weight-gain/weight-loss cycle, mice exhibited a loss in microbial isoflavonoid and steroid biosynthesis metagenomic function that led to exacerbated weight regain, body fat, and lower energy expenditure upon reintroducing HFD. Furthermore, this trait was transmissible where microbiota transfer from cyclic calorically restricted mice to noncyclic mice resulted in greater weight gain and poor glucose tolerance when HFD fed. However, replenishing this microbial metagenomic function through flavonoid administration ameliorated the rate of secondary weight gain after cyclic HFD feeding (169). Together, these studies indicate that preserving a microbial profile and metagenomic function involved in flavonoid metabolism at the nadir phase of body weight loss postcaloric restriction may be an effective strategy in accomplishing long-term weight reduction.

The interaction between caloric restriction/weight loss, changes in the microbiome, and NAFLD is not well understood. Zhang et al. (203) demonstrated in mice that long-term caloric restriction increases *Lactobacillus* with a concomitant...
decrease in LPS-binding protein, a surrogate marker of intestinal permeability, which was independent of diet. This would suggest that prolonged calorie restriction modulates the microbiome to promote intestinal integrity and defend against hepatic insult from microbiota metabolic products. Overall, understanding the clinical implications of calorie restriction in relation to the gut-liver axis and NAFLD is still underdeveloped. Future investigations observing changes in microbial composition and metagenomic function after caloric restriction may provide useful information to its efficacy in improving NAFLD outcomes.

Short- and long-term exercise training. Numerous studies have demonstrated the effectiveness of exercise (3, 48, 74, 82, 90, 139) or physical activity (3, 21, 31, 55, 114, 118, 142, 191, 203) on changing the microbiome. Both forced and voluntary wheel running increased microbial diversity (3, 48, 55, 139), an effect observed even when mice are fed an HFD (48, 55). However, the role of exercise on altering microbiota taxa, particularly the Firmicutes-to-Bacteroidetes ratio, is not as consistent (3, 48, 55, 82). These inconsistencies are likely due to differences in sample location (i.e., small intestine, ileum, cecum, colon, or fecal contents) or method of analysis. Denou et al. (48) used high-intensity interval training and found taxonomic changes differed in each region of the gut, including the Firmicutes-to-Bacteroidetes ratio independently of changes in adiposity. Thus, future studies should evaluate this ratio using other models of exercise training. Despite the inconsistent effect of exercise at the phylum level, genus and species levels of *Bifidobacterium* are consistently shown to be increased with physical activity and exercise (82, 90, 139, 142). *Bifidobacterium* is a known regulator of intestinal permeability (24, 26), suggesting that exercise improves gut barrier function, which is consistent with voluntary wheel running improving intestinal integrity (21). The bidirectional effect of exercise appears to be consistent, but changes in other bacterial species with known metabolic function (e.g., *A. muciniphila*, *B. wadsworthia*, *F. prausnitzii*) are not well described in relation to exercise.

While taxonomic changes in the microbiome with exercise are noted, few studies have related these changes to NAFLD outcomes. Utilizing obese-prone rats fed an HFD with and without voluntary wheel running, Welly and colleagues (191) demonstrated that physical activity-induced alterations in microbiota were associated with improved circulating insulin, LDL cholesterol, liver mass, and liver triglycerides. Interestingly, an unidentified genus in both Rikenellaceae and *Prevotella* were positively correlated with liver triglyceride content (191). These data support a potential role for the ability of exercise to alter the microbiota in the prevention or treatment of NAFLD. Additionally, exercise-induced alterations in the metabolic pathways in the feces may be linked to liver function, including a reversal of the HFD-induced decrease in pathways encoding TCA cycle, glycan biosynthesis, and metabolism, along with carbon fixation (48). Together, these data suggest that the structural alterations to the gut microbiome may be related to the effects seen on reductions in NAFLD risk with exercise training.

**Aerobic fitness and microbiota/NAFLD.** Low cardiorespiratory fitness/aerobic capacity is a strong predictor for mortality and is intimately linked to NAFLD prevalence (125, 171). However, the direct influence of aerobic fitness on changes in the microbiome has not been studied extensively. A recent report in humans with high cardiorespiratory fitness found that VO$_2$max accounted for more than 20% of species richness in fecal samples and individuals with high a VO$_2$max exhibited an increased predicted metabolic function in genes related to bacterial chemotaxis, motility, and fatty acid synthesis (54). It was also found that highly aerobically fit humans had greater fecal butyrate concentrations as well as increased butyrate-producing taxa (i.e., Clostridiales, *Roseburia*, Lachnospiraceae, and Erysipelotrichaceae), compared with low-fitness counterparts with similar body mass, changes that have been linked previously with increased gut health (54).

As described above, exercise is known to change the microbiome in mice and humans, including increases in diversity (i.e., different genera and species of bacteria present), which are linked to improved insulin sensitivity, promotion of satiety, as well as increases in microbial families and genera involved in improved host immunity and metabolism (*Akkermansia* and *Lactobacillus*) (3, 32, 55). In addition, it has been shown that professional athletes have higher α-diversity and distinct clustering in the microbiome, which include lower levels of Bacteroidetes and higher levels of *A. muciniphila* and numerous other taxa, compared with sedentary controls with low or high BMI (32). These are promising observations; however, a cofounder in this analysis was the effect of diet, as athletes consumed much more protein that sedentary controls.

Investigating the impact of aerobic fitness on the microbiome in relation to host metabolism has been studied more closely utilizing rats selectively bred for low capacity running (LCR) and high capacity running (HCR), which intrinsically have differing levels of cardiorespiratory fitness (122, 193). Studies reveal the LCR rats have microbiome profiles that show a greater propensity for energy extraction/harvest from the diet, which may be linked to their poor physiological outcomes, including hepatic steatosis (35, 105). Specifically, Liu et al. (105) demonstrated that exercise decreased Firmicutes and increased Cyanobacteria and Proteobacteria in ovariectomized (OVX) LCR rats, while OVX HCR rats exhibited the opposite effect. Interestingly, Christensenellaceae was greater in HCR vs. LCR rats and positively correlated with running distance, food intake, and body weight (105). In addition, we have recently examined the impact of acute (3 days) HFD on hepatic outcomes in relation to changes in the cecal microbiome of LCR and HCR rats (134). We found that low-fitness LCR rats exhibited a robust increase in hepatic triglycerides, feeding efficiency, and overall positive energy balance compared with HCR rats. These changes in the LCR rats were associated with greater basal relative abundances of SCFA-producing families and genera including *Phascolarctobacterium*, Ruminococcaceae, *Ruminococcus*, and Lachnospiraceae. Predicted metabolic function of the microbiota further suggested that LCRs had a greater capacity to extract energy from the diet, which may have contributed to their increased susceptibility to development of hepatic steatosis. However, in response to acute HFD feeding, LCR rats exhibited a decrease in these SCFA-producing bacteria and showed a strong positive correlation with energy intake and feeding efficiency, relationships that were absent in the HCR rats. These data suggest that poorly aerobically fit LCR rats may lose some of the benefits of SCFA production (i.e., increased satiety) that ultimately increase their susceptibility to HFD-
induced obesity and hepatic steatosis. An alternative hypothesis that is unexplored in this model of aerobic fitness is that there are potential differences in intestinal fat oxidation in low-fitness LCR vs. highly fit HCR rats. Previous work from our laboratory has demonstrated that daily wheel-running exercise can dramatically upregulate the jejunal mRNA expression of several markers of mitochondrial β-oxidation in the OLETF rat model (75), suggesting that fat oxidation in the intestine could potentially impact fat content in the large bowel and influence microbiota composition. Future investigation into intestinal fatty acid metabolism differences between LCR and HCR rats would be an intriguing addition to the knowledge in this model.

Collectively, the available limited evidence provides initial support that the microbiome may be altered in different states of aerobic fitness and these changes may be linked to elevated NAFLD risk seen in humans with low aerobic fitness. Future studies, such as microbiota transplant studies from HCR rats into LCR rats, and vice versa, or humanized transplant studies are warranted and will provide much needed proof of concept for the potential causative role of the microbiome in susceptibility/protection of NAFLD related to changes witnessed with differences in aerobic fitness.

Microbiome and Liver Cancer

The incidence of hepatocellular carcinoma (HCC) is increased with the progression of NAFLD to advanced cirrhosis and may be mediated through changes in the microbiome (116). Fecal *Bifidobacterium* is negatively associated, while *Enterococcus* is positively associated with end-stage liver disease (63). Furthermore, hydrogen peroxide-producing *Lactobacillus* can potentially reverse dysbiosis in end stage liver disease through decreases in *Enterococcus* (63). Yoshimoto et al. (201) found that the secondary bile acid DCA is linked to a senescence-associated secretory phenotype (SASP), which is senescent fibroblasts turning to proinflammatory cells that promote tumorigenesis (34). Specifically, DCA initiates SASP in hepatic stellate cells promoting inflammatory and tumor-initiating mediators that increase the susceptibility to HCC in carcinogen-exposed mice (201). Similar to the progression from NAFLD to NASH, increased LPS and fecal *Escherichia coli* have also been linked to the onset of end-stage liver disease and HCC in humans and rodent models (41, 63, 64, 202). Moreover, chemically induced HCC causes elevated systemic LPS levels, whereas ablation of TLR4, antibiotic supplementation, and also germ-free mice are protected against tumorigenesis (41, 202).

The rise in HCC has been linked to obesity, Type 2 diabetes, and NAFLD, which are replacing viral and alcoholic-induced liver diseases as the major contributors [reviewed in (112)]. Interestingly, initial evidence shows that probiotics can shift the gut microbiome beneficially toward *Prevotella* and *Oscillibacter*, which influence regulation of gut T cell differentiation and alter hepatic proinflammatory cytokines in the extraintestinal tumor microenvironment (100). This is further evidence that the gut microbiome is playing a crucial role in the full spectrum of liver diseases. Overall, this area provides future perspectives on targeting the microbiome throughout all stages of the disease, including steatosis through cirrhosis and HCC.

**Summary**

Figure 3 depicts the overarching principle of how progressing from a “normal” microbiome to “dysbiosis” may have a direct impact on the progression and severity of NAFLD.
Furthermore, lifestyle modifications such as exercise and increasing aerobic fitness, weight loss, and dietary choices can prevent or even reverse dysbiosis, which may prove to be beneficial in the management of NAFLD. Understanding the molecular mechanisms of microbial-derived metabolites on hepatic metabolism is an intriguing area from a pharmaceutical and lifestyle intervention perspective. Overall, we believe that a primary stimulus in the onset and progression of NAFLD is from microbial-derived metabolites and future efforts need to be directed at elucidating their mechanisms for eventual prevention and treatment of the disease.

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DISCLOSURES

No conflicts of interest (financial or otherwise) are declared by the authors.

AUTHOR CONTRIBUTIONS


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