Obesity, Diabetes, and Gut Microbiota

The hygiene hypothesis expanded?

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The connection between gut microbiota and energy homeostasis and inflammation and its role in the pathogenesis of obesity-related disorders are increasingly recognized. Animals models of obesity connect an altered microbiota composition to the development of obesity, insulin resistance, and diabetes in the host through several mechanisms: increased energy harvest from the diet, altered fatty acid metabolism and composition in adipose tissue and liver, modulation of gut peptide YY and glucagon-like peptide (GLP)-1 secretion, activation of the lipopolysaccharide toll-like receptor-4 axis, and modulation of intestinal barrier integrity by GLP-2. Instrumental for gut microbiota manipulation is the understanding of mechanisms regulating gut microbiota composition. Several factors shape the gut microflora during infancy: mode of delivery, type of infant feeding, hospitalization, and prematurity. Furthermore, the key importance of antibiotic use and dietary nutrient composition are increasingly recognized. The role of the Western diet in promoting an obesogenic gut microbiota is being confirmation in subjects. Following encouraging results in animals, several short-term randomized controlled trials showed the benefit of prebiotics and probiotics on insulin sensitivity, inflammatory markers, postprandial incretins, and glucose tolerance. Future research is needed to unravel the hormonal, immunomodulatory, and metabolic mechanisms underlying microbe-microbe and microbiota-host interactions and the specific genes that determine the health benefit derived from probiotics. While awaiting further randomized trials assessing long-term safety and benefits on clinical end points, a healthy lifestyle—including breast lactation, appropriate antibiotic use, and the avoidance of excessive dietary fat intake—may ensure a friendly gut microbiota and positively affect prevention and treatment of metabolic disorders.

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Along with the increasing worldwide incidence of obesity-associated disorders, research has recently unraveled important pathways reciprocally connecting metabolism with the immune system. The development of obesity is a complex process involving genetic susceptibility and environmental factors, which both remain only partially understood. In such instances, gut microbiota is being increasingly recognized as an important factor connecting genes, environment, and immune system. The human gut hosts an enormous number and variety of microorganisms, including at least 10^{14} bacteria belonging to ~1,000 species (1). The genome size of this microbial organ, collectively named microbiome, exceeds the size of the human nuclear genome by two orders of magnitude and provides important biological and metabolic functions that cannot be performed by researchers. Genomic and environmental factors at the basis of mutual host-microbiota interactions have been intensely investigated with metagenomic and metabolomic approaches in the last 5 years. This article will discuss recent advances in understanding the role of gut microbiota in the pathogenesis of obesity, insulin resistance (IR), and diabetes and their potential therapeutic applications.

Evidence for the role of gut microbiota in the regulation of energy homeostasis and fat storage

The first definite evidence for the role of the gut microbiota in the regulation of host energy homeostasis and adiposity came from Gordon and colleagues' (2) group experiments: they noticed that germ-free mice (i.e., raised in the absence of microorganisms) had 40% less total body fat than conventionally raised mice, even if their caloric intake was 29% higher than that of conventionally raised animals (supplementary Table 1, available in the online appendix at http://care.diabetesjournals.org/cgi/content/full/dc10-0556/DC1). In 2 weeks, conventionalization (i.e., colonization of their gut with a cecum-derived, distal microbial community) of germ-free mice produced a 57% increase in total body fat, a 2.3-fold increase in hepatic triglycerides, and a dramatic increase in IR without affecting chow consumption or energy expenditure (2).

In a further key experiment, Backhed et al. (3) fed germ-free or conventionalized mice a high-fat, high-carbohydrate Western diet. After 8 weeks, germ-free mice gained significantly less weight and fat mass than conventionalized mice and were protected against the Western diet-induced glucose intolerance and IR. In contrast to the previous experiment, germ-free and conventionalized mice had similar energy content in their feces, suggesting a more efficient energy harvest from the diet might not be the only factor responsible for the fat mass gain of the conventionalized mice. The investigators also provided a mechanistic basis for the observed resistance of germ-free mice to diet-induced obesity (3):

1) conventionalization doubled the density of small intestinal villi capillaries and enhanced monosaccharide uptake from the gut into the portal blood, stimulated carbohydrate response element binding protein-mediated and sterol responsive element binding protein-1–mediated hepatic and adipose tissue lipogenesis, eventually promoting fat accumulation in the liver and adipose tissue.

2) gut microbiota-promoted storage of circulating triglycerides into adipocytes by suppressing intestinal secretion of an inhibitor of adipose tissue lipoprotein lipase called fasting-induced adipose factor (FIAF), also known as angiopoietin-like protein 4. Consistently, conventionalization of FIAF-deficient knockout (KO) mice produced only a 10% increase

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in total body fat compared with the 57% fat gain observed in wild-type littermates; germ-free FIAF KO mice fed a high-fat, high-carbohydrate diet were not protected from diet-induced obesity (3). Therefore, the blunted FIAF expression might have contributed to triglyceride accumulation in adipocytes and adipose tissue hypertrophy of conventionalized germ-free mice.

3) germ-free mice showed an enhanced activation of hepatic and muscle fatty acid oxidative pathways, which was mediated by two complementary and independent mechanisms: *a*) an increased activity of the enzyme AMP-activated protein kinase, which activates key enzymes of mitochondrial fatty acid oxidation, including acetyl-CoA carboxylase and carnitinepalmitoyltransferase I and *b*) an increased FIAF-induced expression of the nuclear transcription factor peroxisomal proliferator-activated receptor coactivator-1α, a key coactivator of nuclear receptors and enzymes involved in fatty acid oxidation.

Subsequent independent metagenomic and metabolomic studies provided further mechanistic insight into the increased capacity of the obese gut microbiome to harvest energy from the diet:

1) the obese gut microbiome is depleted of genes involved in motility (chemotaxins, motility proteins, flagellar assembly) and enriched in glycoside hydrolases, capable of breaking down otherwise indigestible alimentary polysaccharides; in phosphotransferases involved in the import of simple sugars including glucose, fructose, and *N*-acetylgalactosamine; in β-fructosidase, capable of degrading fructose-containing carbohydrates such as sucrose to lactate, butyrate, or acetate; and in other transport proteins and fermentation enzymes further processing breakdown products (4,5).

2) gut microbiota on a high-fat diet may convert dietary choline into hepatotoxic methylamines, reducing choline bioavailability of choline, which is necessary for the assembly and secretion of VLDLs and eventually promoting hepatic steatosis, IR, and lipoperoxidation (6).

3) multicompartmental top-down metabolic profiling revealed gut microbiota may modulate host hepatic and systemic lipid metabolism through modification of bile acid conjugative patterns, directly impacting on emulsification and absorption properties of bile acids and indirectly impacting on hepatic fat storage and lipoperoxidation through bile acid signaling properties (7).

Collectively, these experiments demonstrated that gut microbiota may modulate both sides of the energy balance equation, namely energy harvest from the diet, energy storage as triglyceride, and energy expenditure through fatty acid oxidation, and that may mediate diet-induced obesity, IR, and diabetes.

Altered gut microbiota composition in obesity: animal and human data

The human gut contains ~1,000 different bacterial species with 99% of the total population belonging to about 40 species (1). The bacterial density progressively increases along the small bowel from $\sim 10^4$ in the jejunum to 10^7 colony-forming units per gram of luminal content at the ileal end, with a predominance of gram-negative aerobes and some obligate anaerobes (8). In the colon, the bacterial count reaches around 10^{12} colony-forming units per gram with a predominance of anaerobes. It has been estimated that 60% of the fecal mass is accounted for by bacteria (8). Despite these observations, research in the field has long been hampered by methodological limitations. Conventional culturing techniques can in fact detect only ~30% of the total intestinal bacteria (8) for several reasons: the unknown growth requirements of the bacteria, the selectivity of the media that are used, the stress imposed by the cultivation procedures, the necessity of strictly anoxic conditions, and the difficulties with simulating the interactions of bacteria with other microbes and host cells (8). Recent culture-independent molecular biologic approaches based on the sequence diversity of the small subunit rRNA (16S rRNA and18S rRNA) gene have overcome these limitations. Fingerprinting techniques, PCR and dot blot hybridization, fluorescent in situ hybridization (FISH), and DNA microarrays substantially enhanced the detection capability of numbers and the diversity of human gut microbiota (9). Although consistently magnifying the insight of investigators into microbial diversity, these techniques each have their own biases and limitations, which should be taken into account when interpreting discrepant results across studies. For instance, FISH depends by its nature on sequence data availability and hence, it fails to detect novel RNA sequences. Furthermore, FISH can miss up to 30% of bacterial cells in a given sample due to either cell permeability or probe mismatch issues (10).

Overall, the application of these molecular techniques revealed that species inhabiting the human gastrointestinal tract are dominated by anaerobic bacteria and belong to three bacterial phyla (divisions): the gram-positive Firmicutes and Actinobacteria and the gram-negative Bacteroidetes. The Firmicutes is the largest bacterial phylum, comprising over 200 genera, including *Lactobacillus*, *Mycoplasma*, *Bacillus*, and *Clostridium* species. The Bacteroidetes (including ~20 genera) and the Actinobacteria also belong to the dominant gut microbiota, but the latter are frequently missed by RNA gene sequencing and can only be detected by FISH (8). To further complicate this picture, the prevalence and diversity of bacteria in different areas of the gastrointestinal tract is influenced by the different conditions at these sites and thus, the microbiota of the stomach and jejunum varies with that of the large intestine.

Animal models suggest obesity is associated with alterations of the composition and the functional properties of the gut microbiota, e.g., the development of obesity in leptin-deficient *ob/ob* mice correlates with a shift in the abundance of the two dominating divisions, Bacteroidetes and Firmicutes. Compared with lean littermates fed the same polysaccharide-rich diet, obesity was associated with a 50% reduction in Bacteroidetes and a proportional division-wide increase in Firmicutes (11).

The relationship between diet, gut microbiota, and energy homeostasis was further investigated in models of diet-induced obesity (4,5), e.g., the microbiota of mice fed a high-fat, high-sugar Western diet was compared with the microbiota of mice receiving a low-fat, high-polysaccharide diet. The Western diet increased the relative abundance of Firmicutes due to a bloom in the class of Mollicutes at the expense of the Bacteriodetes, inducing an enrichment in genes enabling energy harvest from the diet (see above). Importantly, these changes in microbial composition and its functional properties were totally reversed after a shift back to the original diet (supplementary Table 1). To further assess whether diet by itself can affect gut microbiota composition independent of obesity, Hildebrandt et al. (12) employed the resistin-like molecule-β (RELM-β) KO mice, a model that is resistant to high-fat–induced obesity. When RELM-β KO and RELM-β wild-type mice

were switched from a standard diet to a high-fat diet, the changes in the composition and functional properties of the gut microbiome were similar between wild-type and KO mice, indicating that the effects of diet dominated over the obese phenotype.

To definitely demonstrate that altered gut microbiota composition is a cause and not a consequence of obesity or altered dietary habits, caecal microbiota from lean and obese mice was transplanted into the gut of germ-free mice. After 2 weeks, the mice hosting the "obese microbiota" extracted more calories from their food and showed a significantly greater increase in their fat mass than the mice colonized with the "lean gut microbiota" (4,13). These data were independently replicated by other models where the colonization of lean mice by gut microflora extracted from obese animals induced significant fat gain and IR compared with the microbiota extracted from lean animals despite a similar caloric intake (4,5).

Data from human studies were generally consistent with the results from animal models, e.g., 12 obese subjects had lower Bacteroidetes and more Firmicutes in their distal gut than did lean control subjects. After randomization to either carbohydrate-restricted or fat-restricted diets for 52 weeks, the proportion of Bacteroidetes increased over time, mirroring reductions in host weight but not dietary changes (14).

A subsequent metagenomic study (15) with 154 individuals—including monozygotic and dizygotic twins concordant for leanness or obesity and their mothers—also showed that obesity was associated with a markedly reduced bacterial diversity, a relative depletion of Bacteroidetes, and a higher proportion of Actinobacteria compared with leanness. This large-scale study revealed that the human gut microbiome is shared to some extent among family members, but that each person's gut microbial community varies in the specific bacterial lineages present with a comparable degree of covariation between adult monozygotic and dizygotic twin pairs. It also suggested the gut microbiota is inherited to a significant extent from the mothers, and that "inheritance" of the gut microbiota may be more important for microbial community structure and function than the actual genetic context of the host. Examined individuals notably shared a wide array of microbial genes, named a "core gut microbiome" at the gene rather than at the organismal level, comprising an enrichment in phosphotransferases and other carbohydrate-processing and lipid-utilizing genes previously demonstrated in animal models of diet-induced obesity.

Other relatively small studies examined gut microbiota composition in human obesity and type 2 diabetes and the impact of weight reduction on microbial flora. Although generally confirming the above findings, the results were more heterogeneous due to different methodologies and the actual complexity of human lifestyle as compared with experimental animal models, where all potential confounding factors, including the frequency and composition of meals, can be precisely controlled. For these reasons, a definite causal relationship between gut microbiota and the development of obesity remains to be demonstrated in humans (16–21).

Mechanisms linking gut microbiota to obesity, IR, and type 2 diabetes

Beside an increased energy harvest from the diet, further mechanisms linking gut microbiota to obesity have been subsequently proposed, including chronic low-grade endotoxinemia, regulation of tissutal biologically active fatty acid composition and modulation of gut-derived peptide secretion.

Chronic inflammation induced by low-grade endotoxinemia. Metabolic pathways are functionally integrated with immune responses, and the relevance of the innate immune system for the pathogenesis of metabolic disorders is increasingly recognized, e.g., in mice fed a high-fat diet, the activation of liver resident macrophages Kupffer cells promotes hepatic IR and glucose intolerance. The selective depletion of these cells, without affecting adipose tissue macrophages, restores hepatic insulin sensitivity and improves whole-body and hepatic fat accumulation along with glucose metabolism (22,23).

Recent work has shown that gut bacteria can initiate the inflammatory state of obesity and IR through the activity of lipopolysaccharide (LPS), a component of the gram-negative bacterial cell walls, which can trigger the inflammatory process by binding to the CD14 toll-like receptor-4 (TLR-4) complex at the surface of innate immune cells. The relevance of the TLR-4 pathways for metabolic disease was confirmed by the finding that the deletion of TLR-4 prevented the high-fat diet–induced insulin resistance (24).

Cani et al. (25) elegantly demonstrated that after 4 weeks of high-fat feeding, mice exhibited an obese phenotype accompanied by a change in gut microbiota composition (the reduction of Bifidobacteria and Eubacteria spp.) and a two- to threefold increase in circulating LPS levels, which they called "metabolic endotoxemia" since LPS plasma concentrations were much lower than those observed during septic shock. When metabolic endotoxemia was reproduced by subcutaneous infusion of LPS, animals developed the same metabolic abnormalities induced by the high-fat diet, while LPS receptor KO (CD14KO) mice were resistant to the effects of both high-fat diet and LPS infusion. Moreover, CD14KO mice were hypersensitive to insulin even when they were fed a normal diet, suggesting that CD14 may modulate insulin sensitivity in physiological conditions. In a subsequent experiment (26), changes in gut microbiota composition induced by antibiotic treatment reduced metabolic endotoxemia and the cecal content of LPS, closely correlating with an improvement in the obese phenotype in both high-fat-fed and *ob/ob* mice (supplementary Table 1).

The role of LPS in triggering systemic inflammation was subsequently evaluated in healthy human subjects. Anderson et al. (27) found a similar grade endotoxemia increased adipose tumor necrosis factor (TNF)-α and interleukin (IL)-6 concentrations and promoted IR, and a high-fat, high-carbohydrate meal induced a significant postprandial plasma LPS elevation, accompanied by an increased mononuclear cell expression of TLR-4, nuclear factor-κB (NF-κB), and suppressor of cytokine signaling-3 (SOCS-3), an adipokine involved in IR. These increases were totally absent after an American Heart Association (AHA) meal rich in fiber and fruit (28).

Taken together, these data support the concept that endotoxinemia may play a key role in the pathogenesis of obesity-associated inflammatory state and that food ingestion affects plasma endotoxin levels.

Different nutrients have different pro-endotoxinemic potentials. The knowledge of the impact of different nutrients on microbial LPS production or on intestinal LPS absorption could have relevant therapeutic implications. The finding that high-fat feeding reduced the expression of epithelial tight junction proteins occludin and ZO-1, leading to increased intestinal permeability and LPS levels, suggests

intestinal fat absorption and secretion may have a predominant role in LPS entry into the portal blood (47). Consistently, a high-fat diet induced a higher increase in plasma LPS compared with an isocaloric high-carbohydrate diet in mice (29). The ability of fat to induce higher endotoxin levels seems confirmed in recent human studies. In a sample of 201 healthy men, circulating LPS concentrations positively correlated with 3-day total energy and fat, but not with other nutrient intake (29). In healthy subjects, a high-fat meal acutely increases plasma endotoxemia to concentrations that are sufficient to activate cultured human aortic endothelial cells through the release of soluble TNF-α from monocytes (30). Deopurkar et al. (31) compared the effects of an isocaloric meal rich in glucose, saturated fat (cream), or orange juice on plasma endotoxin, oxidative, and inflammatory markers in healthy subjects, and while the expression of NF-κB, SOCS3, TNF-α, and IL-1β increased significantly following glucose and cream intake, plasma LPS concentrations and TLR-4 expression increased only after cream intake. Orange juice did not change any of the indexes measured, and, when added to a high-fat, high-carbohydrate meal, it prevented postprandial increase in plasma endotoxin, TLR-4, and related inflammatory markers (31,32) (supplementary Table 1).

Another dietary pattern that has been linked to both metabolic disorders and endotoxinemia is excessive fructose intake. Mice consuming high-fructose solution for 8 weeks showed a 27-fold increase in portal endotoxin levels, coupled with a significant increase in plasma inflammatory cytokines, hepatic steatosis, and IR, compared with water controls. These alterations, except increased portal endotoxin levels, were markedly blunted in fructose-fed TLR-4–mutant mice, further confirming the LPS-TLR-4 axis may mediate the deleterious metabolic effects of excessive fructose intake (33).

Collectively, these data suggest different nutrients have different abilities to induce an endotoxinemic and inflammatory response with fat and possibly fructose having the greatest potential. Plasma endotoxin increase may derive from enhanced LPS production by microbiota or from increased intestinal LPS absorption. Unfortunately, little is known about mechanisms regulating LPS absorption. Ghoshal et al. (34) showed that endotoxin is actively secreted into the blood along with the formation and secretion of chylomicrons in animals and cultured enterocytes and is not just translocated due to the breakdown of the intestinal barrier, and that inhibiting chylomicron formation blocked LPS secretion. These findings suggest that the inhibition of chilomicron secretion may effectively reduce metabolic endotoxinemia and may ultimately benefit obesity-associated metabolic disorders, even in the absence of overt hyperlipidemia.

Other modulators of gut microbiota composition. Growing evidence suggests factors other than dietary habits can modulate gut microflora and that the 1st years of life have a crucial impact on the individual's gut microbiota composition. In a prospective study (35), children becoming overweight by 7 years of age had lower levels of Bifidobacteria and higher levels of *Staphylococcus aureus* during the 1st year of life than infants maintaining a healthy weight. Another study (17) found that the response of overweight adolescents to a diet and exercise weight-loss program was dependent on the initial microbiota prior to the treatment.

While not taking into account confounders such as various nutrient intake, these studies suggest the knowledge of factors modulating gut microbiota composition early in life may have therapeutic or preventive implications for adult obesity.

The fetus is sterile in uterus and is colonized by microbes during its passage through the birth canal. Immediately after birth, the baby is exposed to several environmental sources of bacteria (e.g., skin, mouth, mother's milk). This initial microbiota changes dynamically during the first months of life, owing to the continuous exposure to different environmental bacteria. Gut microbiota has fully matured by the first 1–2 years of life, coinciding with the weaning from the high-fat milk diet to the solid high-carbohydrate diet, and thereafter remains substantially constant throughout the individual's life and fluctuates around a core of stable colonizers (36,37). Results from the KOALA (Kind, Ouders en gezondheid: Aandacht voor Leefstijl en Aanleg) Birth Cohort and other studies have suggested the mode of delivery, type of infant feeding, hospitalization, prematurity, and antibiotic use determine the gut microbial composition during infancy (38–40). During a natural birth, infants are rapidly colonized by microbes from the mother's birth canal and feces, while babies delivered by cesarean section are colonized by environmental microbes from their mother, the air, and transferred by the nursing staff. As a result, infants delivered by cesarean section have fewer intestinal Bifidobacteria and *Bacteroides* spp. (two species shown to be protective against obesity) and are more often colonized by *C. difficile* in comparison with vaginally delivered infants.

Formula-fed infants are more often colonized with *Enterobacteriaceae* spp., *C. difficile*, *Bacteroides* spp., and *Streptococcus* spp. compared with breast-fed infants who are predominantly colonized by *Staphylococcus* spp., *Streptococcus* spp., Lacobacilllus spp., and Bifidobacterium spp. Whether different gut microbe colonization explains the different propensity for obesity from different infant feeding requires further studies with careful prospective monitoring of gut flora and lifestyle habits.

The pervasive impact of antibiotic use on gut microbes is also increasingly recognized, e.g., a 5-day course of oral antibiotics modifies human gut microbiota for up to 4 weeks before it tends to revert to its original composition, and some communities fail to recover within 6 months (41). Consistently, the use of antibiotics in infants is associated with the decreased number of the antiobesogenic *Bifidobacteria* and *Bacteroides*, and after antibiotic treatment there is a slow regrowth of *Bifidobacteria*, whereas *Bacteroides* spp. are not usually reestablished (38).

Collectively, these findings highlight the importance of nondietary factors in determining the composition of gut microflora.

Regulation of adipose tissue and liver fatty acid composition by gut microbes

Gut microbiota can also affect host metabolism and inflammatory state by modulating the tissue fatty acid composition: mammalian intestinal Lactobacilli and Bifidobacteria can synthesize from free linoleic acid bioactive isomers of conjugated linoleic acid, which have antidiabetic, anti-atherosclerotic, immunomodulatory, and anti-obesity properties (42). The supplementation of Bifidobacterium breve and linoleic acid to different mammalian species resulted in a two- to threefold higher intestinal, hepatic, and adipose tissue content of *cis*-9, *trans*-11 conjugated linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid, concomitantly with a reduced proinflammatory cytokines TNF-α, IL-6, and interferon-γ ex-

pression, than the linoleic acid-alone supplemented diet (43) (supplementary Table 1).

Gut microbiota modulates gut-derived peptide secretion

PYY. Gut microbiota synthesizes a large amount of glycoside hydrolases that break down complex plant polysaccharides to monosaccharides and short-chain fatty acids, mainly acetate, propionate, and butyrate. Beside representing an important source of energy for de novo lipogenesis, these short-chain fatty acids are ligands for two G-protein–coupled receptors, Gpr41 and Gpr43, of gut enteroendocrine cells (44). Upon ligand binding, these G-protein–coupled receptors stimulate secretion of PYY, which inhibits gut motility and slows intestinal transit thereby enhancing nutrient absorption. Consistent with these properties, conventionally raised Gpr41-deficient mice or germ-free Gpr41-deficient mice colonized with *Bacteroidetes theatiotaomicron* and *Methanobrevibacter smithii* (two common commensals of human distal gut) were significantly leaner than wild-type littermates, whereas there were no genotype-related differences in germ-free mice. Gpr41 deficiency was associated with decreased expression of PYY, faster intestinal transit rate, and reduced harvest of energy from the diet (44) (supplementary Table 1).

GLP-1 secretion. Gut microbiota fermentation of prebiotics promoted L-cell differentiation in the proximal colon of rats and increased glucagon-like peptide (GLP)-1 response to a meal in healthy humans (45,46). *Ob/ob* mice treated with prebiotic carbohydrates had altered gut microbiomas and increased circulating GLP-1 and GLP-2 (47). Further supporting the relevance of GLP-1 in mediating prebiotic action, genetic or pharmacological deletion of GLP-1 prevented the beneficial effects of prebiotics on weight gain, glucose metabolism, and inflammatory pathway activation (48,49).

GLP-2 secretion. Recent experimental data suggest gut microbiota may modulate gut barrier integrity and endotoxinemia through GLP-2, a 33-amino acid peptide with known intestinotrophic properties, which is cosecreted with GLP-1 by enteroendocrine L-cells.

Cani et al. (47) assessed the effect of the prebiotic fermentable oligofructose on gut microbiota composition, intestinal permeability, and hepatic and systemic inflammation in *ob/ob* mice. Compared with the carbohydrate-alone diet, the prebiotic + carbohydrate diet increased the intestinal proportion of Lactobacilli and Bifidobacteria, preserved tight junction integrity and intestinal barrier function, and lowered endotoxinemia and systemic and hepatic cytokines and oxidative stress. These effects were associated with an increased intestinal GLP-2 production, were abolished by the pretreatment with a GLP-2 antagonist, and were mimicked by the administration of a GLP-2 agonist (47), thus suggesting GLP-2 may mediate the benefits of prebiotics.

Gut microbiota manipulation: human trials

Following the encouraging results in animals (50,51), the effects of manipulating enteric flora by probiotics (live bacteria given in oral quantities that allow for colonization of the colon) or prebiotics (nondigestible oligosaccharides like insulin and oligofructose that are fermented by colonic microbiota and enhance the growth of beneficial commensal organisms like *Bifidobacterium* and *Lactobacillus* spp.) have been evaluated in several controlled trials (supplementary Table 2). The randomized controlled trials (RCTs) did not exceed 6 months' duration, were mostly relatively small-sized (<50 participants), and evaluated surrogate markers rather than clinical end points, which further substantiated the mechanisms of action of pre/probiotics formerly elucidated in animals, e.g., increased satiety and reduced caloric intake by prebiotics, enhanced GLP-1 and PYY responses, and reduced glucose excursions and inflammatory responses postprandially (52–54).

The three largest RCTs evaluated the effect of probiotics on pregnancy outcomes and perinatal growth patterns. The impact of perinatal probiotic administration on the development of overweight and obesity was assessed in a follow-up study from birth to 10 years of age (55), e.g., 159 pregnant women were randomized to receive probiotics (*Lactobacillus rhamnosus*) or placebo from 4 weeks prior to delivery through 6 months after delivery. The perinatal probiotic intervention was safe and moderated weight gain during the first 1–2 years of life, but did not affect the second phase of excessive weight gain starting after 24–48 months of age. The intervention also showed a trend to reduce the birth weight-adjusted mean BMI at 4 years of age.

Two RCTs assessed the effect of maternal probiotic-supplemented dietary counseling on pregnancy outcome, glucose regulation, and perinatal growth, e.g., 256 women were randomized in the 1st trimester of pregnancy to receive nutritional counseling or as control subjects; the dietary intervention group was further double-blindly randomized to receive probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium lactis*) or placebo (diet/placebo), while the control group received placebo (control/placebo) (56,57). Overall, probiotic supplementation was safe with blood glucose concentrations and homeostasis model assessment index during pregnancy and over the 12-month postpartum period the lowest in the diet/probiotics group, which also had a reduced incidence of gestational diabetes mellitus. No significant differences in prenatal or postnatal growth rates among the study groups were detected, but dietary intervention diminished the risk of larger birth size in affected cases.

Conclusions

Numerous animal models consistently demonstrated that gut microbiota can modulate host energy homeostasis and adiposity through different mechanisms, e.g., energy harvest from the diet, LPS-induced chronic inflammation, modulation of tissue fatty acid composition, and gut-derived peptide secretion. Although extensive experimental data suggested gut microbiota manipulation can beneficially affect host adiposity and glucose metabolism, a causal relationship between gut microbes and obesity still needs to be proven in humans, in whom current data suggest an association between gut microbiota, Western diet, and obesity. In the only follow-up study (35) prospectively connecting gut microbiota to the development of obesity, other factors, including dietary habits, were not assessed, making causal inference uncertain. The assessment of pro/prebiotic efficacy in free-living humans is far more complex than under standardized experimental conditions because different confounding factors, including antibiotic use, background diet and physical activity, endotoxin content of ingested food, and even meal frequency (58), may affect gut microbiota, energy balance, and ultimately body weight. Understanding these factors may allow researchers to design future trials and better understand the relative impact of pre/probiotics on the treatment of obesity, which is a complex disease deriving from the interaction of largely un-

known multiple genetic and environmental factors. The ongoing double-blind, randomized, controlled trial, FATLOSE, is assessing the effect of healthy donor feces transplantation on glucose homeostasis and intestinal inflammation in subjects with metabolic syndrome and will hopefully help address these issues. Furthermore, the long-term safety of gut microbiota manipulation needs assessment. We are used, in fact, to consider probiotics a safer alternative or a complement to drugs, but the impact of prolonged perturbation in gut microbial ecology is unknown, as currently only one RCT specifically assessed the safety of probiotic supplementation for as long as 6 months (59).

Future research should also move beyond profiling human gut microbial species and focus on the functional properties ensuring health benefits for the host. Toward this aim, it will be essential to elucidate the complex mechanisms of action of pre/probiotics, which are only lately being unraveled. These include the production of direct antimicrobial substances (bacteriocins); the competition for the same biological niche and prevention of replication of other communities (colonization resistance); the stimulation of production of antibacterial substances, including mucins by epithelial cells and defensins by crypt Paneth cells; the modulation of the epithelial cell proinflammatory transcription factor NF-κB; and the stimulation of mucosal B-cell and T-cell immunity to produce secretory immunoglobulin A, proinflammatory (i.e., IL-12), or anti-inflammatory (i.e., IL-10) cytokines (60). Importantly, most effects are strain-specific and varying probiotic strains can exert different and even opposite anti- or proinflammatory actions. These data emphasize the need for a deeper knowledge of molecular mechanisms underlying microbe-microbe and microbe-host interactions to tailor a more selective approach targeting the individual needs.

While awaiting well designed RCTs with clinical end points, the importance of a "healthy" lifestyle in its broader sense—including breast lactation, a healthy diet, avoiding excessive fat, appropriate antibiotic use—cannot be overemphasized and may ensure a friendly gut microbiota, positively affecting metabolic outcomes. A concept that can be drawn from available studies is that the nutrient value of food is a relative and not an absolute term that can be influenced by our microbiome metabolic activity. Conversely, our food choices may imprint into inner intestinal metabolome by affecting the structure and activity of the gut microbiota. This metabolic imprinting starts at an early stage of life and metagenomic studies could allow researchers to obtain a deeper understanding of the nutritional needs of humans and yield microbiome-based biomarkers for identifying those at risk for obesity. The understanding of factors modulating gut microbiota assembly early in life may have preventive implications for adult obesity. Under an evolutionary perspective, the obesity epidemic can be viewed as an extension of the hygiene hypothesis: the data presented suggest that improved sanitation and living conditions, overzealous antimicrobial therapy, and Westernized dietary patterns in developed countries may predispose to metabolic diseases just as improved hygiene increased the susceptibility to allergic and autoimmune diseases, and that a deviant gut microbiota may mediate these associations.

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