Obesity, diabetes, and gut microbiota: the hygiene hypothesis expanded?

This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/81901 since

Terms of use:
Open Access
Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)
Obesity, Diabetes, and Gut Microbiota

The hygiene hypothesis expanded?

GIOVANNI MUSSO, MD 1
ROBERTO GAMBINO, PHD 2
MAURIZIO CASSADER, PHD 2

The connection between gut microbiota and energy homeostasis and inflammation and its role in the pathogenesis of obesity-related disorders is 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 microbiota 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 confirmed 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.

Diabetes Care 33:2277–2284, 2010

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 a large number 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 definitive 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...
Gut microbiota and obesity

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 carnitine palmitoyltransferase 1 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-acetyl-galactosamine; 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 hepatoxic 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) multicompartamental 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 ~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 and 18S rRNA) gene have overcome these limitations. Finger-printing 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 on 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-poly saccharide diet. The Western diet increased the relative abundance of Firmicutes due to a bloom in the class of Mollicutes at the expense of the Bacteroidetes, 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 co-variation 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 endotoxemia, regulation of tissue/sex-linked activity fatty acid composition and modulation of gut-derived peptide secretion.

**Chronic inflammation induced by low-grade endotoxemia.** 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, revinces 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 theecal content of LPS, closely correlating with an improvement in the obes 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 endotoxemia 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-endotoxemic 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 endotoxin 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 rich in glucose, saturated fat (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 endotoxemia 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-TRL-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 chylomicron secretion may effectively reduce metabolic endotoxemia 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., *Lactobacillus* 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 re-growth 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 Lactobacillus and *Bifidobacteria* can synthesize from free linoleic acid bioactive isomers of conjugated linoleic acid, which have antidiabetic, antiatherosclerotic, 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 expression.
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 *Bacteroides thetaiotamoticron* 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 microbiomes 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 endotoxemia through GLP-2, a 33-amino acid peptide with known intestinotrophic properties, which is cosecreted with GLP-1 by enterodendocrine 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 endotoxemia 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 (non-digestible 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 (<30 participants), and evaluated surrogate markers rather than clinical end points, which further substantiated the mechanisms of action of pre/probiotics formally 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-blindedly 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 benefically 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 antibiotic 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.

Acknowledgments—This work was supported in part by grants from the Piedmont Regional Funds Comitato Interministeriale per la Programmazione Economica 2008. No potential conflicts of interest relevant to this article were reported.

G.M. researched the data, discussed the data, and wrote the manuscript. R.G. researched the data and contributed to the discussion. M.C. contributed to the discussion and reviewed the manuscript.

References
1. Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology 2009;136:65–80
2. Backhed F, Ding H, Wang T, Hooper LV, Koh GT, Nagy A, Semenkovich CF, Gordon JJ. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101:15718–15723
3. Backhed F, Manchester JK, Semenkovich CF, Gordon JJ. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A 2007;104:979–84
4. Turnbaugh PJ, Backhed F, Fulton L, Gordon JJ. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 2008;3:213–223
5. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JJ. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 2009;1:1–10
6. Dumas ME, Barton RH, Toy A, Cloarec O, Blancher C, Rothwell A, Fearnside J, Tatour R, Blanc V, Lindon JC, Mitchell SC, Holmes E, McCarthy MI, Scott J, Gauquier D, Nicholson JK. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci USA 2006;103:12511–12516
7. Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, Lek P, Rezzi S, Ramadan Z, van Bladeren P, Fay LB, Kochhar S, Lindon JC, Holmes E, Nicholson JK. Probiotic modulation of symbiotic gut microbiota: host-microbiome interactions in a humanized gnotobiotic mouse model. Mol Syst Biol 2008;4:1–13
8. Zoetendal EG, Vaughan EE, de Vos WM. A microbial world within us. Mol Microbiol 2006;59:1639–1650
9. Andoh A, Benno Y, Kanauchi O, Fujiyama Y. Recent advances in molecular approaches to gut microbiota in inflammatory bowel disease. Curr Pharm Des 2009;15:2066–2073
10. Ley RE. Obesity and the human microbiome. Curr Opin Gastroenterol 2010;26:5–11
11. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JJ. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 2005;102:11070–11075
12. Hildebrandt MA, Hofmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F, Wu GD. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology 2009;137:1716–1724.e1–2
13. Turnbaugh P, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JJ. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–1031
14. Ley RE, Turnbaugh P, Klein S, Gordon JJ. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022–1023
15. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egelheim M,Henissart B, Heath AC, Knight R, Gordon JJ. A core gut microbiome in obese and lean twins. Nature 2009;457:480–484
16. Duncan SH, Lobley GE, Holtrop G, Ince J, Johnstone AM, Louis P, Flint HJ. Human colonic microbiota associated with diet, obesity and weight loss. Int J Obes 2008;32:1720–1724
17. Santacruz A, Marcos A, Wärnberg J, Marti A, Martin-Matillas M, Campoy C, Moreno LA, Veiga O, Redondo-Figuer D, Garagorri JM, Azcona C, Delgado M, Garcia-Fuentes M, Collado MC, Sanz Y. EVASYON Study Group. Interplay be-
between weight loss and gut microbiota composition in overweight adolescents. Obesity (Silver Spring) 2009;17:1906–1915.

18. Nadal I, Santacruz A, Marcos A, Warnberg J, Garagorri M, Moreno LA, Martin-Martíllas M, Campoy C, Martí A, Molerès A, Delgado M, Veiga OL, García-Fuentes M, Redondo CG, Sanz Y. Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. Int J Obes 2009;33:758–767.

19. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braudot M, Yu Y, Parameswaran P, Crowell MD, Wing R, Ruttmann BE, Krajmalnik-Brown R. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci USA 2009;106:2365–2370.

20. Wu X, Ma C, Han L, Nawaz M, Gao F, Zhang X, Yu P, Zhao C, Li L, Zhou A, Wang J, Moore JE, Millar BC, Xu J. Molecular characterisation of the faecal microbiota in patients with type II diabetes. Curr Microbiol 2010;61:69–78.

21. Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, Al-Soud WA, Sørensen SJ, Hansen LH, Jakobsen M. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS One 2010;5:e9085.

22. Neyrinck AM, Cani PD, Dewulf EM, De Backer F, Bindels LB, Delzenne NM. Critical role of Kupffer cells in the management of diet-induced diabetes and obesity. Biochem Biophys Res Commun 2009;385:351–356.

23. Huang W, Metlakunta A, Dedousis N, Ghanim H, Abuaysheh S, Knauf C, Bastelica D, Neyrinck AM, Fava S, Lecourt E, Dewulf EM, Sohet R, Kalliömaäki M, Collado MC, Salminen S, Delzenne NM, Burcelin R. Improvement of diet-induced diabetes and insulin resistance. Diabetes Care 2009;32:2281–2287.

24. Shi H, Kokoeva MV, Inouye K, Tzameli I, Huang W, Metlakunta A, Dedousis N, Delzenne NM, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:3353–3358.

25. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmete ÉE, Cousin B, Sulipe TC, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Castella M, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761–1772.

26. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57:1470–1481.

27. Anderson PD, Mehta NN, Wolfe ML, Hinkle CC, Pruscoino L, Comiskey LS, Tabita-Martínez J, Sellers KF, Rickels MR, Ahima RS, Reilly MP. Innate immunity modulates adipokines in humans. J Clin Endocrinol Metab 2007;92:2272–2279.

28. Ghanim H, Abuaysheh S, Sia CL, Korzenevskii K, Chaudhuri A, Fernandez-Real JM, Dandonà P. Increase in plasma endotoxin concentrations and the expression of toll-like receptors and suppressor of cytokine signaling-3 in monocellular cells after a high-fat, high-carbohydrate meal: implications for insulin resistance. Diabetes Care 2009;32:2281–2287.

29. Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, Chamontin B, Ferriere J. Energy intake is associated with endotoxaemia in apparently healthy men. Am J Clin Nutr 2008;87:1219–1223.

30. Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. Am J Clin Nutr 2007;86:1286–1292.

31. Depopurkar R, Ghanim H, Friedman J, Abuaysheh S, Sia CL, Mohanty P, Viswanathan P, Chaudhuri A, Dandonà P. Differential effects of cream, glucose and orange juice on inflammation, endotoxin, and the expression of toll-like receptor-4 and suppressor of cytokine signaling-3. Diabetes Care 2010;33:991–997.

32. Ghanim H, Sia CL, Upadhayay M, Korzenevskii K, Viswanathan P, Abuaysheh S, Mohanty P, Dandonà P. Orange juice neutralizes the proinflammatory effect of a high-fat, high-carbohydrate meal and prevents endotoxin increase and toll-like receptor expression. Am J Clin Nutr 2010;91:940–949.

33. Spruss A, Kanuri G, Wagnerberger S, Haub S, Bischoff SC, Berghelm I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. Hepatology 2009;50:1094–1104.

34. Ghoshal S, Witta J, Zhong J, de Villiers W, Wall R, Ross RP, Shahidi, F, O’Mahony L, O’Mahony C, Coakley M, Hart O, Lawlor P, Quigley EM, Kiely B, Fitzgerald GF, Stanton C. Metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. Am J Clin Nutr 2009;89:1393–1401.

35. Samuel BS, Shaito A, Mutoke T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon J. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 2008;105:16767–16772.

36. Cani PD, Hoste S, Guoy T, Delzenne NM. Dietary non-digestible carbohydrates promote I-cell differentiation in the proximal colon of rats. Br J Nutr 2007;98:32–37.

37. Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikhan BD, Naslawn D, De Backer F, Neyrinck AM, Delzenne NM. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. Am J Clin Nutr 2009;90:1236–1243.

38. Cani PD, Possemiers S, Van de Wiele T, Guoy T, Everard A, Rottier O, Geurts L, Naslawn D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58:1101–1103.

39. Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R. Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide-1 receptor. Diabetes 2006;55:1484–1490.

40. Zhou J, Martin RJ, Tulley RT, Raggio AM, McCutcheon KL, Shen L, Danna SC, Tripathy S, Hegsted M, Keenan MJ. Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner
50. Membrez M, Blancher F, Jaquet M, Bibiloni R, Cani PD, Burcelin RG, Corthesy I, Macé K, Chou CJ. Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. FASEB J 2008;22:2416–2426

51. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM. Selective increases of bifidobacteria in gut microbiota improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 2007;50:2374–2383

52. Parnell JA, Reimer RA. Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults. Am J Clin Nutr 2009;89:1751–1759

53. Nilsson AC, Ostman EM, Holst JJ, Björck IM. Including indigestible carbohydrates in the evening meal of healthy subjects improves glucose tolerance, lowers inflammatory markers, and increases satiety after a subsequent standardized breakfast. J Nutr 2008;138:732–739

54. Solga SF, Buckley G, Clark JM, Horska A, Diehl AM. The effect of a probiotic on hepatic steatosis. J Clin Gastroenterol 2008;42:1117–1119

55. Luoto R, Kalliomäki M, Laitinen K, Isolauri E. The impact of perinatal probiotic intervention on the development of overweight and obesity: follow-up study from birth to 10 years. Int J Obes (Lond). 16 March 2010 [Epub ahead of print]

56. Laitinen K, Poussa T, Isolauri E, Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota Group. Probiotics and dietary counselling contribute to glucose regulation during and after pregnancy: a randomised controlled trial. Br J Nutr 2009;101:1679–1687

57. Luoto R, Laitinen K, Nermes M, Isolauri E. Impact of maternal probiotic-supplemented dietary counselling on pregnancy outcome and prenatal and postnatal growth: a double-blind, placebo-controlled study. Br J Nutr 2010;103:1792–1799

58. Crawford PA, Crowley Jr, Sambandam N, Muegge BD, Costello EK, Hamady M, Knight R, Gordon JI. Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. Proc Natl Acad Sci USA 2009,27:11276–11281

59. Allen SJ, Jordan S, Storey M, Thornton CA, Gravenor M, Garaiova I, Plummer SF, Wang D, Morgan G. Dietary supplementation with lactobacilli and bifidobacteria is well tolerated and not associated with adverse events during late pregnancy and early infancy. J Nutr 2010;140:483–488

60. Garrett WS, Gordon JI, Glimcher LH. Homeostasis and inflammation in the intestine. Cell 2010;140:859–870