EMERGING ROLES OF THE GUT MICROBIOTA IN THE PATHOGENESIS OF METABOLIC DISORDERS

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EMERGING ROLES OF THE GUT MICROBIOTA IN THE PATHOGENESIS OF METABOLIC DISORDERS

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EDITORIAL
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Editorial: Emerging Roles of the Gut Microbiota in the Pathogenesis of Metabolic Disorders

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Editorial on the Research Topic

Emerging Roles of the Gut Microbiota in the Pathogenesis of Metabolic Disorders

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The human gut is inhabited by a highly complex and metabolically active microbial ecosystem composed by trillions of members. The gut microbiota heavily influences host physiology through microbiota-derived molecules (1). Variations in its composition induce metabolic changes that may result in alterations of host phenotype, being its metabolic activity essential in maintaining host homoeostasis and health. Moreover, gut microbes can also lead to severe metabolic disorders. On the other hand, gut microbiome is highly sensitive and can be altered throughout lifespan mostly by environmental factors. The host can affect the microbial ecosystem through its immune system, genetic background, sex, and age. All these factors may induce gut microbiota imbalances that are often associated with metabolic alterations.

Metabolic disorders are defined as a group of diseases where normal metabolic processes are disrupted due to the accumulation of large amounts of one metabolite or a deficiency of one or more metabolites. Metabolic disorders have a variety of clinical presentations ranging from acute symptoms in the neonatal period to slower, more gradual onsets at a later age. Metabolic diseases can be inherited or acquired during the lifetime. Inherited metabolic disorders result from a genetic defect in functioning of an intermediate metabolic pathway, while acquired disorders are resulting from external factors, with lifestyle factors as main causes.

The present Research Topic highlights the interplay between host metabolism and gut microbiota. Overall, it focuses on microbiota composition, functionality and metabolites in the pathogenesis of metabolic disorders and on the effect of microbiota modulation on host metabolism.

In recent years, a substantial body of literature has provided evidence for the link between gut microbiota and diabetes, disease that affects more than 463 million people worldwide (2). Several articles in the present topic focus on the relation between gut microbiota and the most prevalent type of diabetes, type 2 diabetes (T2D). A large cohort of T2D patient study presented by Diener et al. provides a potential set of four bacterial genera as a biomarker of T2D disease progression and risk.

Moreover, they found that the four identified genera returned to near-normal levels in T2D treated individuals. In this line, a systematic review by Cao et al. analysed the reciprocal interactions between gut microbiota and anti-hyperglycemic drugs, focusing on the effect of the gut microbiome on diabetic control *via* bug-host interactions. They found that changes in specific taxa and β-diversity of gut microbiota were associated with metformin and acarbose in humans. A review by Huda et al. reports the recent findings regarding the role of gut microbiota in T2D, focusing on the causal relationship between microbiota and T2D. They not only summarized the associations between T2D and microbial metabolites but also described how host genetic architecture and the epigenome influence the microbial composition. Finally, they discussed future directions in this field, pointing at the potential of faecal microbiota transplantation, prebiotics, and probiotics supplementation as therapeutics for T2D. One of the most frequent and severe microvascular complications of diabetes is diabetic kidney disease. Fang et al. summarized in their review the current findings regarding the role of gut microbial metabolites in the development and progression of diabetic kidney disease.

Another type of diabetes, gestational diabetes mellitus (GDM) affects around 7–10% of all pregnancies worldwide (3). Soderborg et al. suggest a potential influence of GDM alone and together with maternal overweight/obesity on infant microbiota in patterns that set the stage for future risk of inflammatory and metabolic disease. Moreover, An et al. have explored the problem of small-sized newborns finding that gut microbiota may play a role on metabolic disorders during this critical period, particularly through *Lactobacillus* and short-chain fatty acids (SCFAs).

The review of Martín-Núñez et al. compiled the latest evidence from human studies on the influence of *Helicobacter pylori* infection and its eradication therapies on the composition of the gut microbiota and host's metabolic health. The effect of altered microbiota on metabolic disorders is clearly shown in several studies, however, it is intriguing that modulating metabolism can itself influence the composition of microbiota in the gut. Huang et al. showed in their article the effects of a 6-week program of training exercise and dietary restriction on gut microbiome composition, metabolism and central hemodynamic parameters in obese adolescents. They have found that exercise and diet interventions significantly reduced body weight, levels of glucose, triglycerides and HDL cholesterol, and improved measures of central hemodynamics, which correlated with altered gut microbiota. From a different point of view, Massier et al. reviewed the underlying mechanisms of an impaired intestinal barrier and its possible impact on metabolic health. They focus on recent findings on how endotoxemia and translocation of bacteria, bacterial genetic material and products may cause host dysfunction subsequently contributing to metabolic diseases. The authors concluded that it seems unavoidable that microbiota also contribute to the modulation of their metabolic environment shaping the body's responses to nutrients and contributing ultimately to disease. This latter conclusion is of immense importance in the design and application of future therapies as inadequate research of interventional drug therapies could fire back through undesired modulation of the metabolic environment or the compounds produced by the microbiota itself.

The example of an inherited metabolic disease has been represented by the work by van der Goot et al. who used the metabolic disease phenylketonuria to discuss how microbial ecology and eco-evolutionary aspects within a challenged gastrointestinal tract lead to microbial relationships that can be used to prevent neurological problems through the fascinating gut-brain axis.

Within the scientific community there is no doubt about the fact that alterations in gut microbiota constitute an important factor in the development of metabolic disorders. Moreover, current lifestyles in the developed countries are foreboding that metabolic disorders due to deranged gut microbiota will not decrease any time soon. Therefore, understanding the role of gut microbiota in the pathogenesis of metabolic disorders may hold exciting prospects for the treatment of these diseases. However, the interplay is still poorly understood which makes real breakthroughs in therapeutic interventions less feasible. Taken together, to delineate the exact mechanisms of this interplay, different research fields must combine their expertise to tackle this important question.

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OPINION
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A Microbial Community Ecology Perspective on the Gut-Microbiome-Brain Axis

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The gut microbiome is the collection of all microbial cells and associated genetic material present in the digestive tract of a host. Its composition depends on the gut environment and nutrition provided by the host (1). In turn, the microbes help the host by metabolizing complex nutrients, protecting against pathogens and priming their immune system (1, 2). In fact, the influence of the gut microbiome on host development goes beyond nutrition and immune response, by also regulating host behavioral and neurological responses (3, 4).

Microbiome-associated phenotypes can be seen as means for adaptation and natural selection, presenting an accessible point on which selection could work to tweak host-phenotype in case of changes in environmental conditions (5). From an animal perspective, the microbiome-associated phenotypes gained interest when Bercik et al. showed that fecal matter transplantation could direct strain-specific behavior of the recipient toward strain-specific behavior of the donor (6). Since then, a growing body of evidence has supported the effects of the microbiome on brain and behavior and the concept of a gut-microbiome-brain axis [see for reviews (3, 4)].

Although the microbiome-gut-brain-axis has been implicated in the pathophysiology of various (mental) diseases (3, 4), it is often an overlooked aspect in many (metabolic) disorders associated with behavioral deficits and treatments on a dietary basis. Which is remarkable, as diet is one of the main determinants of the gut microbiome and affects the (development) of cognitive (dys)function (7, 8). Moreover, beneficial effects of probiotic treatment on cognition have been reported for pathological conditions such as irritable bowel syndrome and coeliac disease (9).

Despite the growing interest in gut microbiome, the ecological aspects associated with these microbial communities are often not considered in the interpretation of the data, although they might contribute to the great variability in host performance often observed after microbiome manipulation, potentially leading to inconclusive interpretation of the data (10, 11). For instance, as communities, the gut microbiome is not static, being subjected to large fluctuations that reflect interactions among resident and transient microbial species and the host. From an ecological perspective, these interactions are driven by rules associated with microbial succession, as they reflect changes in community composition in response to processes such as selection and drift (12). Moreover, approaches targeting microbiome manipulation, including the development of probiotics (the "good" bacteria) or prebiotics (food for probiotics), require unraveling the ecological principles controlling microbial invasion, where mechanisms associated with microbial diversity and resource competition can help predicting the outcome of these strategies (11).

Despite the potential of using microbiome-related strategies to improve current treatment and neurological outcome of (metabolic) disorders, we argue that unraveling the ecological

principles associated with the community dynamics is crucial, and subsequently a prerequisite to ensure the success in microbial-based treatments. To put our opinion in context, we will use the metabolic disorder phenylketonuria (PKU) to illustrate and explain how general concepts of community dynamics and resource availability, eco-evolutionary aspects and microbial invasions, apply in situations where the environment of the gastrointestinal tract is challenged and diet or probiotics are used to prevent neurological problems.

THE GUT MICROBIOME IN THE CONTEXT OF PKU

PKU is an enzymatic deficiency of the hepatic phenylalanine hydroxylase which results in dramatically increased levels of Phe (>600 μmol/L). It can reach levels that are considered to be toxic for the brain, leading to severe intellectual disability (13–15). The most common treatment is to restrict the intake of natural protein in the diet, thus preventing high Phe levels in the brain, while supplementing with amino acids and essential micronutrients to avoid deficiencies (15–17). When followed early and continuously, this treatment is very effective in keeping Phe levels within an acceptable range (360–600 μmol/L), preventing severe high-Phe associated intellectual disability (15). Nevertheless, in many PKU patients with normal cognitive function Phe levels still influence brain performance (18). Alternatively, it has been shown that inconsistencies in neurocognitive, psychosocial and metabolic consequences of PKU remain, despite treatment (19–22).

It has been shown, that elevated Phe levels are not only present in the blood and brain, but are also manifesting in the gut (23). The microbes in the gut environment are constantly competing for resources, which become available either through host nutrition or the by-products generated by the microbial chemical food webs (24, 25). Together, the selective pressure exerted by the available resources and biotic interactions pressure play important roles in determining the microbiome composition in the gut of a given host (12, 26, 27). This means that the microbiome of PKU patients is constantly challenged by situations ranging between two extremes, depending on adherence to treatment; untreated with altered amino acid profiles and high Phe-levels, or low Phe-levels accompanied by a change in resource availability due to the strict dietary requirements. As the microbiome is adaptive in nature, this is likely to result in an altered microbial community. In both situations, alterations are likely to cause a less diverse microbiome, as Phe has been shown to be toxic to certain cell types (neurons), and resource restriction (natural protein) will challenge microbial species that either rely on these resources or are vulnerable to it, making it less likely to establish or to be successful (survival). Results from both PKU mice and patients show that, indeed, the PKU-associated microbiome is often less diverse and more variable between individuals, indicating dominance of a few species within a community (28–31). Moreover, studies that have examined prebiotic supplementation in PKU infant formula or the prebiotic properties of medical foods (glycomacropeptide) showed promising results in maintaining or increasing microbial diversity, indicating that altered resources might influence microbiome diversity in PKU (30, 32).

From a microbial ecology standpoint, the consequences of a less diverse microbiome include the community susceptibility to disturbances. Ecological theories predict that high diversity acts a biotic barrier, contributing to a stable microbiome or promoting microbiome resilience, capable of returning to the original, healthy state, upon disturbance. Thus, a reduction in gut microbial diversity significantly limits the ability of the microbiome to withstand major shifts, potentially leading to alternate, diseased, stable states (33, 34).

Another importance consequence of reduction in gut microbiome diversity or shifts in composition in PKU patients is the associated changes in the metabolite profiles of the microbiome, potentially modulating the chemical food web, thus influencing stability, as well as the molecules involved in microbiome-gut-brain signaling and brain functioning (35–38). In general, although many functions carried out by the microbiome show functional redundancy, i.e., that multiple populations are capable of carrying out that function, variability in the observed function after microbiome manipulation is greater than the change in gene frequency (10). This means that the interactions within the community strongly impact the functionality of the microbiome. As these functions include production of neurological signaling molecules, a dysfunctional microbiome could lead to behavioral symptoms. In case of dietary treated pathological conditions like PKU, the functionality of the microbiome could therefore impact the neurocognitive, psychosocial and metabolic outcome, despite a highly demanding diet. To optimize the outcome, and thus improving quality of life, a promising alternative would be the development of probiotics that could promote microbial diversity and microbiome functionality. Such approaches have been successfully used in other metabolic diseases (obesity related insulin insensitivity and type 2 diabetes), which are also associated with a dysbiotic gut microbiota and reduced microbial complexity (39, 40).

ECO-EVOLUTIONARY ASPECTS ASSOCIATED WITH PROBIOTIC SUPPLEMENTATION

Probiotic supplementation for PKU can serve two purposes—it can be used to escape behavioral problems associated with treated PKU and it can be utilized to lower absorption of Phe from the gut by utilizing microbial metabolism. Although it has been shown that colonization is not necessary for probiotic action, interactions with the commensal microbiota will make supplementation less controllable and might lead to the unpredictable effects (41). Colonization of a desired probiotic or microbial consortia will therefore lead to longer lasting and more reproducible effects. Thus, for successful probiotic supplementation, general concepts such as community

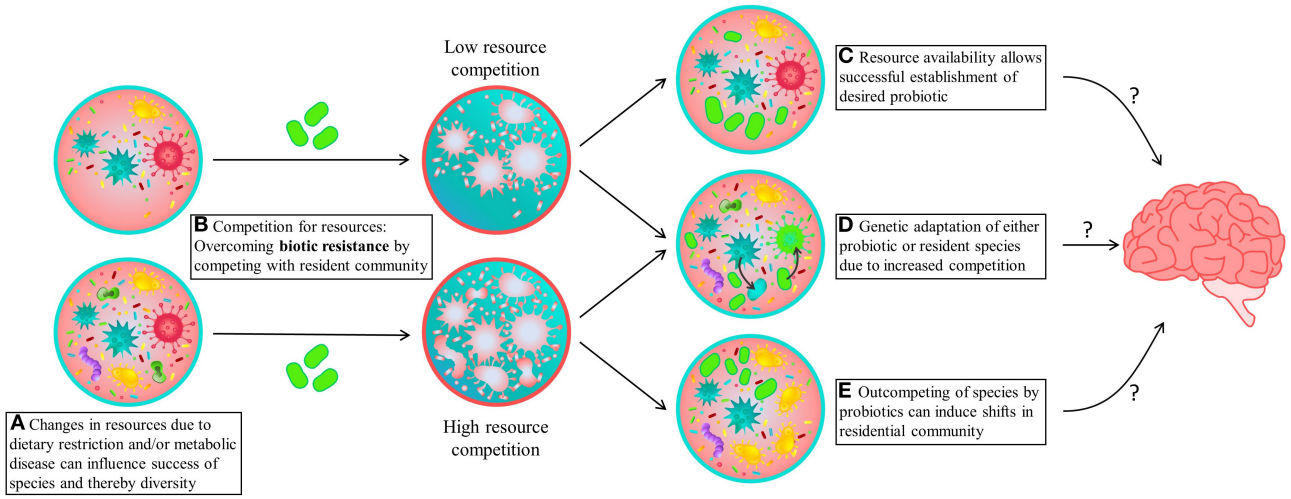

FIGURE 1 | A schematic overview of potential consequences of probiotic supplementation. **(A)** An altered resource availability due to dietary restriction. **(B)** When presenting a probiotic (indicated by green microbes), these species will have to compete for available resources (represented by the blue color). **(C)** When the microbial diversity is low, resource availability will be sufficient for successful establishment and growth of the desired probiotic. However, when the microbial diversity is high, it usually correlates with high resource utilization and thus lower resource availability for the invading probiotic, leading to high competition and lack of establishment. This increased competition can either lead to **(D)** (genetic) adaptation of either the probiotic or resident species and **(E)** permanent or transient changes in the composition and functionality of the resident species, which in case of keystone species could lead to dramatic shifts in microbial community. These community dynamics could therefore lead to unpredictable changes in microbiome composition and potentially different effects on brain functioning then initially predicted from *in vitro* studies.

dynamics, microbial invasions and colonization are important to take into consideration to increase treatment effectiveness and safety.

From an ecological perspective, probiotic supplementation can be studied in the context of microbial invasions, where the probiotic non-indigenous strain is introduced in large numbers into an existing community [(11, 42); **Figure 1**]. For microbial invasion to be successful, the invader has to overcome both abiotic (i.e., environmental factors like pH and temperature) and biotic resistance imposed by the resident community (**Figure 1B**). Ecological theory predicts that effectiveness and success of a probiotic treatment depends on the ability of the probiotic strain to invade and colonize the gut—which is correlated with their high growth rates, phenotypic plasticity and genetic diversity—but also depends on its capacity to compete for resources in the presence of the native gut microbiome (11, 43, 44). It has been shown that the success of invasion—in this case, the establishment of a probiotic strain—is negatively correlated with microbiome diversity (11, 45, 46). Specifically, diverse microbial communities explore the metabolic resources available in the gut in a more efficient manner, thus limiting the number of niches available for invaders (probiotic) to get established. On the other hand, when food resources are not fully consumed and invading species are capable of utilizing empty niches, the chances of establishment and growth are high (**Figure 1C**). In PKU, dietary restrictions and/or the influence of changes in amino acid profiles are likely responsible for lower species diversity due to altered resource availability, as opposed to liberalized dietary restrictions (**Figure 1A**). Thus, from the PKU perspective, the observed low gut microbiome diversity might increase the chances of probiotic establishment, although the altered resource availability intrinsic to PKU diet could prevent establishment and survival of the desired probiotics. Additional strategies that increase the probiotic's competitive ability, such as the use of prebiotics that stimulate the growth of the probiotic strain, or high phenotypic plasticity that ensure quick adaptation, might increase the chances of successful colonization therefore improving treatment effectiveness.

Examining microbiome dynamics during colonization (whether is it permanent or not) or whether the microbes were prone to adapt (mutate) within the gut-environment is important to determine safety of a given probiotic (47). In PKU, recent scientific advances give rise to the use of genetically modified probiotics to lower the absorption of Phe from the gut by relying on microbial metabolism (47–50). However, although they have shown to be stable as probiotics, little is known about how these supplements affect the ecological and evolutionary dynamics of commensal microbiota. Even with successful and beneficial introduction of the probiotics, the effects on the resident community, and subsequently behavior, might be more unpredictable—an aspect that is inherent to all microbial invasions (34, 51). Moreover, in situations in which the invader needs to compete for available resources, this increases the selective pressures and the propensity for horizontal gene transfer, which could lead to adaptation of either the probiotic or resident species (**Figure 1D**) (52–55). In the context of genetically modified bacteria, as for instance the Phe lowering probiotic, this could lead to integration of the modification in other (commensal) species, risking unfavorable

expression of the given genes. Additionally, recent developments have shown that the probiotic properties of certain probiotic strains might be attributed to genes that induce mutational patterns that increase the risk of developing colorectal cancer (56). Thus, a systematic search for naturally occurring gut microbiome strains capable of degrading Phe might represent a more sustainable solution toward personalized medicine, where the evolutionary principles of the gut microbiome are considered (57).

Lastly, ecological principles associated with community dynamics might influence the outcome of microbiome manipulations due to the intricate relationship among microbial populations and their chemical food web (34, 51). Due to these community dynamics and its effect on resource availability, successful invasions can displace or shift resident taxa and alter community function, affecting multiple connections within the network (25, 58, 59). For instance, loss of a keystone species, one that is responsible for many connections in a chemical network, would make the community prone to collapse or create dramatic shifts in composition and function (**Figure 1E**). Microbiome alterations due to pre- or probiotics can therefore lead to changes in bacterial (metabolite) profile, resulting in different effects than predicted by *in vitro* studies, and to large impacts on the functionality of the entire microbiome. This could also explain why microbiota transfer therapy shows promising results, whereas supplementation of pre- and probiotics leads to variable results (60, 61). Unraveling the dynamics on composition, functionality and potential interactions within the gut microbiome are thus crucial to developing successful microbial based treatments and predicting its effects on microbiome functioning and cognitive outcome.

CONCLUDING REMARKS

Microbial-based strategies for relieving neurological symptoms in various disorders are currently being studied more extensively. Nevertheless, additional research is needed to gain insight in the evolutionary and ecological microbial community dynamics. These dynamics play a critical role in the stability, composition and functional diversity of our gut microbiota, and thus the safety and success of probiotic treatment. Moreover, these ecological principles might explain the discrepancies found between animal and human studies, where results in the often more complex (human) microbiota are less profound and do not lead to the desired outcomes after microbiome manipulation (62, 63). Examining bacterial metabolite profiles resulting from the microbiome could uncover the exact mechanisms by which the gut microbiome influences the brain and hence many behavioral domains. It then could be used to develop personalized probiotic supplementation for disorders requiring dietary treatment, including PKU.

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EG, JF, and EZ came up with the original concept and wrote the paper. EG had the primary responsibility for the final content. All authors read and approved the final manuscript as submitted and contributed in designing the final concept.

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Effects of Non-insulin Anti-hyperglycemic Agents on Gut Microbiota: A Systematic Review on Human and Animal Studies

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Background: As growing evidence links gut microbiota with the therapeutic efficacy and side effects of anti-hyperglycemic drugs, this article aims to provide a systematic review of the reciprocal interactions between anti-hyperglycemic drugs and gut microbiota taxa, which underlie the effect of the gut microbiome on diabetic control via bug-host interactions.

Method: We followed the PRISMA requirements to perform a systematic review on human vs. animal gut microbiota data in PubMed, SCOPUS, and EMBASE databases, and used Cochrane, ROBIN-I, and SYRCLE tools to assess potential bias risks. The outcomes of assessment were trends on gut microbiota taxa, diversity, and associations with metabolic control (e.g., glucose, lipid) following anti-hyperglycemic treatment.

Results: Of 2,804 citations, 64 studies (17/humans; 47/mice) were included. In human studies, seven were randomized trials using metformin or acarbose in obese, pre-diabetes, and type 2 diabetes (T2D) patients. Treatment of pre-diabetes and newly diagnosed T2D patients with metformin or acarbose was associated with decreases in genus of *Bacteroides*, accompanied by increases in both *Bifidobacterium* and *Lactobacillus*. Additionally, T2D patients receiving metformin showed increases in various taxa of the order *Enterobacteriales* and the species *Akkermansia muciniphila*. Of seven studies with significant differences in beta-diversity, the incremental specific taxa were associated with the improvement of glucose and lipid profiles. In mice, the effects of metformin on *A. muciniphila* were similar, but an inverse association with *Bacteroides* was reported. Animal studies on other anti-hyperglycemic drugs, however, showed substantial variations in results.

Conclusions: The changes in specific taxa and β-diversity of gut microbiota were associated with metformin and acarbose in humans while pertinent information for other anti-hyperglycemic drugs could only be obtained in rodent studies. Further human studies on anti-hyperglycemic drugs other than metformin and acarbose are needed to explore gut microbiota's role in their therapeutic efficacies and side effects.

Keywords: anti-hyperglycemic drugs, microbiome, microbiota, association, systematic review

INTRODUCTION

Gut microbiota plays a pivotal role in the pathogenesis of diabetes as significant alterations were found in the gut microbiome composition in type 2 diabetes (T2D) patients relative to healthy individuals (1). A metagenome-wide association study reported a moderate degree of dysbiosis associated with depletion in butyrate-producing bacteria, accompanied by increases in opportunistic pathogens among diabetic patients (2). These changes were echoed by a recent systematic review, which shows an inverse association of T2D with the genera *Bifidobacterium, Akkermansia* and butyrate-producing bacteria (e.g., *Roseburia, Faecalibacterium*), in conjunction with a positive association with *Ruminococcus, Fusobacterium,* and *Blautia* (1).

From a clinical perspective, these findings provide a rationale for targeting gut microbiota imbalance as a potential strategy for T2D treatment by restoring a healthy gut microbiome, including fecal microbiota transplant and probiotic supplements (3, 4). However, the efficiency and effectiveness of these treatments remain uncertain due to concerns over the invasive nature of fecal microbiota transplant and the dosage, species, and duration required for an effective probiotic treatment. Emerging evidence indicates that the therapeutic efficacy of anti-hyperglycemic drugs might, in part, be attributable to their ability to modulate the compositions of gut microbiota (1, 3, 5–9). This compositional change might lead to enrichments in bacterial species exhibiting beneficial effects to intestinal health *via* the production of health-promoting metabolites, such as short-chain fatty acids (SCFAs) and bile acids (8). Nevertheless, certain anti-hyperglycemic drugs were reported to cause increases in the abundance of *Escherichia* and *Candidatus Arthromitus*, which contribute to gastrointestinal side effects and weight gain, respectively (9–11).

Among various anti-hyperglycemic drugs in clinical use, metformin, acarbose, sitagliptin, and vildagliptin (5, 6) have been investigated for their reciprocal interplay with gut microbiota by assessing their effects on human and animal gut microbiota, and *vice versa* (1, 8, 11). From a translational perspective, animal models might help to explore the causality of complex host-microbiota interactions and possible mechanisms of action in a controlled experimental setting. However, it should be noted that differences in dietary habits, host metabolism, inflammatory states, and body anatomy contribute to great variations in gut microbiota compositions between humans and animals, and subsequently, the respective drug effects in disease control (12). A meta-analysis of published 16S rDNA sequencing data from mouse and human fecal microbiota showed that there were significant increases in *Lactobacillus* and *Turicibacter* genera in mouse gut microbiota while the genera of *Streptococcus, Ruminococcus, Lachnospira, Faecalibacterium, Dialister*, and *Oscillospira* were elevated in human gut microbiota (12). Moreover, age, mouse strains/populations, microbiota pools in laboratories, and other practical factors might have varied to a great extent among different studies (12). Previous reviews have suggested the effects of anti-hyperglycemic drugs on gut microbiota (8, 11, 13), however, the differences in results between human and animal studies have not been differentiated.

Reciprocal interplays between individual anti-hyperglycemic drugs and gut microbiota remain unexplored with respect to the contribution of specific bacterial taxa to drug's therapeutic efficacy in disease control (i.e., the clinical question). Thus, we conducted this systematic review aiming to shed light on the associations among anti-hyperglycemic agents, changes in specific taxonomic groups of gut microbiota, and host glucose control or metabolic profiles mainly in humans, as compared to those reported in animal studies.

METHODS

Literature Search

Our literature search strategies were designed to integrate the following PICOS (population, intervention, comparisons, outcomes, study design) based on the prior clinical question: Population: humans (e.g., healthy people or patients who were either obese, prediabetes, diabetes) or the corresponding animal models; Intervention: non-insulin anti-hyperglycemic drugs; Comparisons: post- vs. pre-intervention, with-vs. without-treatment, or on-vs. off-treatment; Outcomes: alteration of the gut microbial composition; Study design: clinical trials, observational studies or animal experiments, as that recommended for systematic reviews (14). We systematically searched PubMed, EMBASE, and SCOPUS databases from January 1, 2000, to November 13, 2019. The keywords and searching strategies based upon the PICOS were "anti-hyperglycemic drugs" and "gut microbiota" related terms (**Supplementary Table 1**). In addition, we searched manually the reference list of the review papers for additional publications of interest.

Study Selection Criteria

We followed the preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines (15). The inclusion criteria for the published studies included: (i) any human studies or animal experiments reporting original data of gut microbiota

after receiving anti-hyperglycemic drugs; (ii) gut microbiota data were analyzed from feces or colonic content specimens; (iii) must be written in English or Chinese. Studies were excluded if they did not provide data of individual bacterial taxa or were only available as conference abstracts or proceedings.

Selection of Studies

Initially, the abstracts and titles of potential articles were screened, followed by the evaluation of the full-text articles for eligibility. Two authors were responsible for screening and evaluating these papers independently. Disagreements were resolved by consensus between these two authors and, if necessary, discussed by additional two authors.

Data Extraction

A standardized form in a Microsoft Excel file (e.g., characteristics of studies, participants, treatments and comparisons, methods to analyze the microbiome, and measures of outcomes) was used for data extraction. Data were extracted by one author and reviewed by a second one. All disagreements were resolved by consensus and a third or fourth author when necessary.

Quality Assessment

We used the Cochrane risk-of-bias tool to assess the risk of bias in selected randomized trials (16). For quasi-experimental and observational studies, we used the Risk of Bias in Non-randomized studies-of Interventions (ROBIN-I) to assess the risk of bias (17). Further, the SYRCLE's risk of bias tool for animal studies (18) was used to assess the risk of bias. The risks of bias data were extracted by four different authors and all disagreements were resolved by consensus made by the remaining authors.

Outcomes of Assessment

Other than describing the characteristics of the evaluated human or animal studies, the primary outcome was the difference in relative abundance or change patterns of individual intestinal bacterial taxa, categorized based on six common taxonomic categories [Phylum (P)c, Class (C), Order (O), Family (F), Genus (G), Species (S)], in associations with the use of anti-hyperglycemic agents, among those available human or animal studies, respectively. Secondary outcomes were differences in microbial diversity, changes in intestinal or serum levels of SCFAs and/or bile acids in human or animal hosts, respectively, after taking/using individual drugs, associations between specific taxa and host metabolic parameters, e.g., glucose, body weight, and lipid profile.

Data Synthesis

We classified the primary and secondary outcomes into the following categories: significant increase, significant decrease, and no significant difference between comparison groups. Changes of each taxon were synthesized from at least 2 studies for human or animal studies, respectively. Specifically, the effects of different anti-hyperglycemic drugs on specific taxon among the evaluated human or animal studies were compared. Further, the corresponding effects of each individual drug on specific taxon were compared to explore its consistency, in terms of having the same trend of alteration on the specific taxon caused by the same specific anti-hyperglycemic drug, or not. These findings also were categorized by the target research populations (e.g., obese, pre-diabetic, newly T2D, prevalent T2D), individual treated drugs or different animal models (mice or rat models with various diets or genetic knockout). For gut microbial diversity, each study might use one or more measures to assess α- (richness and evenness) or β-diversity. We considered α-diversity as "Increase" if at least one measure showed an increase and no measure showed a decrease; "Decrease" if at least one measure showed a decrease and no measure showed an increase; "No difference" if all measures showed no difference. β-diversity was assessed as "Difference" if at least one measure showed a difference; "No difference" if all measures showed no difference. In terms of associations between specific taxa and host metabolic parameters, we collected data from specific taxa that increased or decreased significantly in participants receiving non-insulin anti-hyperglycemic drugs. Only data with statistical significance were extracted for analyses.

RESULTS

The following presented results were mainly focused on human studies, which are compared to those of animal studies.

Reviewed Studies

Overall, 2,804 citations were retrieved, and the final analysis included 17 human studies (7, 19–34) and 47 mouse studies (5, 6, 35–79) from 64 papers (**Figure 1**). The majority of studies were published in and after 2017 and the duration of anti-hyperglycemic treatments varied from studies to studies (i.e., from few days to several months). Of 17 human studies, seven (41.2%) were randomized control trials (7, 19–21, 32–34). Thirteen studies (76.4%) enrolled either newly diagnosed or prevalent T2D patients (20, 21, 23, 24, 26–34), whereas the remaining four studies enlisted healthy participants (22, 25), obese individuals (19), and pre-diabetic patients (7) (**Tables 1**, **2**).

Of 47 rodent studies, 30 (63.8%) studies were conducted in mice (35–41, 44–46, 48, 52, 53, 55, 56, 59–65, 68, 70, 74–79) and the others were in rats (5, 6, 42, 43, 47, 49–51, 54, 57, 58, 66, 67, 69, 71–73). Their characteristics, housing, acclimatization, and diet treatments were presented in **Table 3** and **Supplementary Table 2**. Overall, 14 anti-hyperglycemic agents were used in these included studies, with metformin accounting for the most. There was only one human study focused on glipizide, while the other ten listed drugs (e.g., voglibose, miglitol, vildagliptin, sitagliptin, saxagliptin) were used only in rodent studies (**Table 1**).

Microbiome Assessment Method

Fecal specimens of all human studies were analyzed for the composition of gut microbiota. Of 47 mouse studies, 34 studies (72.3%) used fecal samples (5, 6, 35, 36, 38, 41, 42, 44–46, 49–56, 59–61, 63–73, 76, 78). 16S rRNA gene sequencing was the most common method used in human and animal studies (**Table 1**).

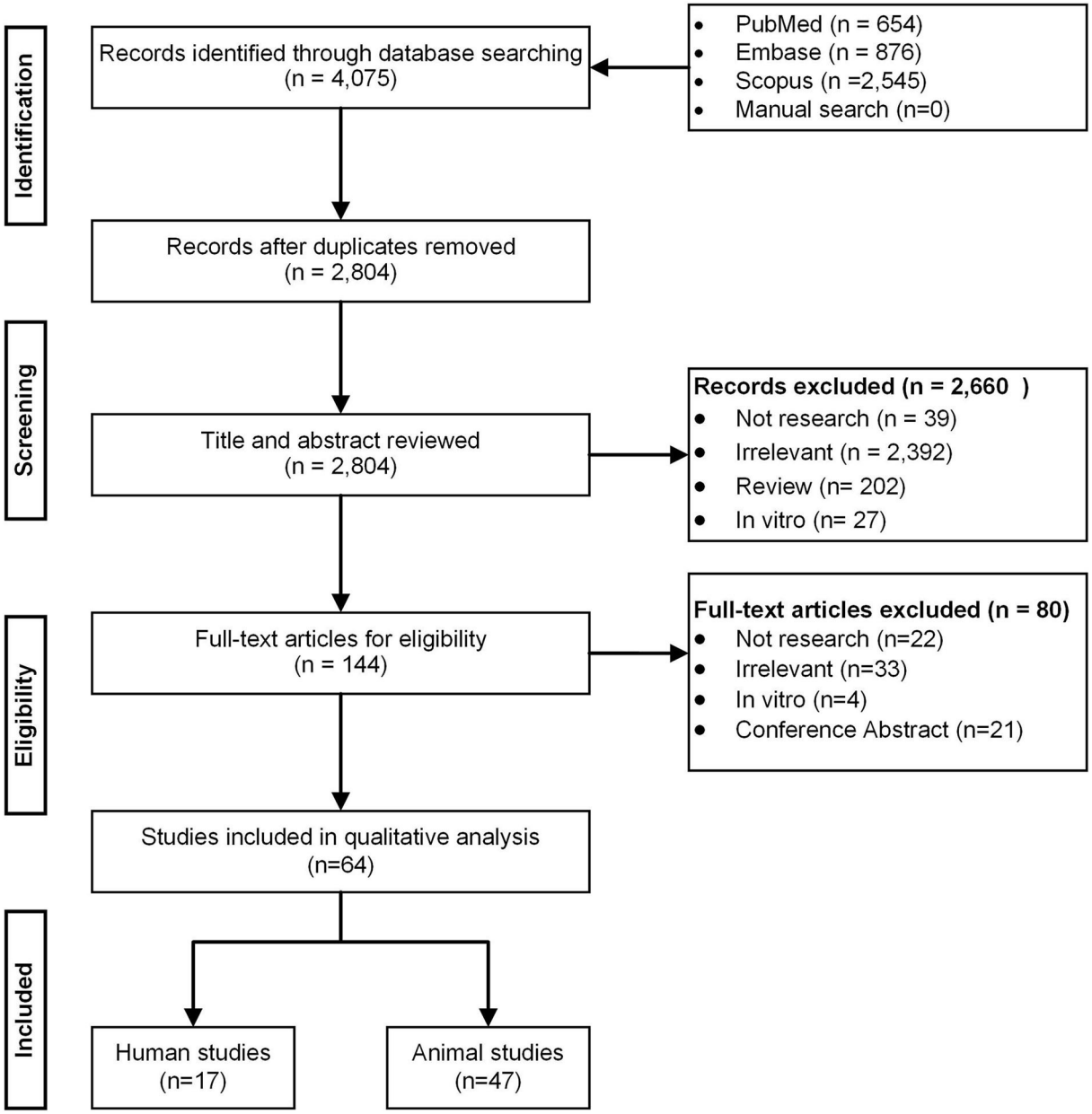

FIGURE 1 | PRISMA flow diagram.

The Risk of Bias

Among human studies, three randomized studies were at the high risk of bias in performance, detection, and attrition, while four studies were unclear risks in most domains (**Supplementary Figure 1**). Among quasi-experimental studies, three studies were at low risk of bias in all domains, whereas two studies were at serious risk in confounding, selection of participants, and classification of interventions (**Supplementary Figure 2**). All 5 cross-sectional studies were at serious risk in several domains, e.g., confounding, selection of participants, classification of intervention (**Supplementary Figure 3**). Almost all mouse studies were unclear risk across domains, even if some were at low risk of bias in selective outcome reporting (**Supplementary Figure 4**).

Outcomes of Assessment

Bacterial Taxa

Importantly, the synthesized results from animal studies reported all common six taxonomic categories (P, C, O, F, G, S) of bacterial taxa but there were only three common taxonomic categories (F, G, S) of gut microbiota taxa were reported in those from human studies based on the available data (**Tables 4**–**7**). Glipizide and

TABLE 1 | General characteristics of all included studies.

Characteristics		Human studies (*N* = 17)		Animal studies (*N* = 47)	
		n	%	*n*	%
Study design	Randomized trials	7	41.2	–	–
	Quasi-experimental studies	5	29.4	–	–
	Cross-sectional studies	5	29.4	–	–
	Animal experiments	–	–	47	100
Treatment					
Biguanide	Metformin	14	82.4	24	51.1
α-glucosidase inhibitors	Acarbose	3	17.6	6	12.8
	Voglibose	–	–	2	4.3
	Miglitol	–	–	1	2.1
GLP-1 receptor agonists	Liraglutide	1	5.9	7	14.9
DPP-4 inhibitors	Sitagliptin	–	–	4	8.5
	Vildagliptin	–	–	2	4.3
	Saxagliptin	–	–	1	2.1
	Anagliptin	–	–	1	2.1
SGLT-2 inhibitors	Dapagliflozin	–	–	2	4.3
	Canagliflozin	–	–	1	2.1
Thiazolidindiones	Pioglitazone	–	–	1	2.1
	Rosiglitazone	–	–	1	2.1
Sulfonylure	Glipizide	1	5.9	–	–
Published year	2019	3	17.6	14	29.8
	2018	7	41.2	16	34.0
	2017	4	23.5	6	12.8
	2016	–	–	5	10.6
	2011–2015	3	17.6	6	12.8
Geography	Asia	7	41.2	33	70.2
	Europe	7	41.2	5	10.6
	North America	2	11.7	8	17.0
	South America	1	5.9	1	2.1
Participants					
Human	Newly T2D	4	23.5	–	–
	Prevalent T2D	9	52.9	–	–
	Healthy	2	11.8	–	–
	Obese	1	5.9	–	–
	Pre-diabetic	1	5.9	–	–
Mice/rats	Diet or STZ or both	–	–	29	61.7
	Gene knockout	–	–	12	25.5
	Diet and gene knockout	–	–	2	4.3
	Wild type with normal diet	–	–	6	12.7
	Other (adenine)	–	–	1	2.1
Specimens	Feces	17	100	34	72.3
	Intestinal, colon, cecal contents	–	–	11	23.4
	Feces and intestinal, colon contents	–	–	2	4.3
Assessment methods	T-RFLP	1	5.9	–	–
	RT-qPCR	1	5.9	6	12.8
	Metagenomic sequencing	5	29.4	–	–

(Continued)

TABLE 1 | Continued

Characteristics		Human studies (*N* = 17)		Animal studies (*N* = 47)	
		n	%	*n*	%
	16S rRNA gene sequencing	10	58.8	35	74.4
	16S rRNA gene sequencing and metagenomic	–	–	1	2.1
	16S rDNA gene sequencing	–	–	3	6.4
	DGGE	–	–	1	2.1
	Cultivation	–	–	1	2.1
Variable gene region for gene sequencing	V3–V4	2	11.8	15	31.9
	V4	3	17.6	9	19.1
	V3	1	5.9	4	8.5
	V1–V2	1	5.9	3	6.4
	V1–V3	–	–	5	10.6
	V1, V2, V3	1	5.9	–	–
	V3–V5	1	5.9	–	–
	V4–V5	–	–	1	2.1
	V5–V6	–	–	1	2.1
	Not stated	1	5.9	1	2.1

T-RFLP, terminal restriction fragment length polymorphism; RT-qPCR, Real-time quantitative polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; –, no information.

liraglutide were assessed in a single human study. No differences were found in patients treated with glipizide (32), while Wang et al. (34) found the association between liraglutide treatment and the increased abundance of genus *Akkermansia* in T2D patients. The assessments of the effects of metformin and acarbose on the human gut microbiota composition represented the foci of 14 studies (19–31, 34) and three studies (7, 32, 33), respectively (**Tables 4**, **5**).

Of the phylum *Bacteroidetes*, the genus *Bacteroides* decreased in two studies treated with metformin among newly diagnosed T2D patients (20, 24), and in two studies treated with acarbose among pre-diabetic and newly diagnosed T2D patients (7, 32). Additionally, one study treated with metformin (24) and one study treated with acarbose (32) in newly diagnosed T2D patients reported similar results of decreases in seven species (e.g., *Bacteroides dorei, Bacteroides finegoldii*). For the phylum *Firmicutes*, the genus *Lactobacillus* increased in two studies in pre-diabetic (7) and newly diagnosed T2D patients (32) receiving acarbose, and the species *L. gasseri* increased in one study treated with metformin (21), and with acarbose (32), respectively, among newly diagnosed T2D patients. Meanwhile, the genus *Clostridium* decreased in one study among healthy participants receiving metformin (22) which was also reported in newly diagnosed T2D patients treated with acarbose (32) (**Table 4**). Two species, i.e., *C. bartlettii* and *C. botulinum*, consistently decreased among T2D patients receiving metformin in two separate studies (21, 31). Three out of four studies showed a decrease in the genus *Intestinibacter* (21, 22, 30) among healthy participants and T2D patients treated with metformin (**Table 5**). With respect to the phylum *Actinobacteria*, the genus *Bifidobacterium* with the species *B. adolescentis* increased in one study with metformin (21) and another study with acarbose (32) among newly diagnosed T2D patients (**Table 4**), and *B. longum* consistently increased among T2D patients treated with acarbose in two studies (32, 33) (**Table 5**).

Concerning other phyla, two studies evaluated the genus *Fusobacterium* (phylum *Fusobacteria*) (20, 27) and the species *Akkermansia muciniphila* (phylum *Verrucomicrobia*) (21, 29) among T2D patients treated with metformin. Both showed increases in the abundance of these two taxa. In the phylum *Proteobacteria*, conflicting results were reported in two studies with respect to the family *Enterobacteriaceae* in healthy participants vs. T2D patients treated with metformin (25, 27). Overall, metformin might increase different taxa from the family *Enterobacteriaceae* and other families in the order of *Enterobacteriales*. Of six studies that evaluated the genus *Escherichia* (19–22, 25, 30) and four studies evaluating the genus *Shigella* (19, 20, 22, 25), the treatment of healthy participants, obese individuals, and T2D patients with metformin all led to increased abundance of these two genera. Another two studies on metformin in newly diagnosed and prevalent T2D patients (21, 31) reported consistently increases in eight species of the order *Enterobacteriales*, including *Citrobacter koseri, Escherichia coli*, *Klebsiella pneumonia* (family *Enterobacteriaceae*), *Erwinia amylovora* (family *Erviniaceae*), *Pectobacterium wasabiae*, and *Dickeya dadantii* (family *Pectobacteriaceae*) (**Table 5**).

The effects of 13 anti-hyperglycemic drugs on the compositions of gut microbiota were conducted in different rodent models. The results of pioglitazone was inconclusive (78), while the other 12 drugs were presented in **Tables 6**, **7**. The *Firmicutes/Bacteroidetes* ratios were decreased in two studies treated with metformin in high fat diet (HDF)-fed mice (37, 48).

TABLE 2 | Characteristics of included human studies.

References, country	Participants	*N*	Treatment and daily dose	Duration	Specimen	Analysis method	Comparison	Outcomes				
								α-diversity	β- diversity	Taxonomic composition	SCFAs	Bile acids
Randomized trials (*n* = 7)												
Ejtahed et al. (19), Iran	Obese	20/16	M (1,000 mg) vs. placebo	2 m	Feces	16S rRNA	Post vs. pre	√	√	√	√	
Tong et al. (20), China	Newly T2D	100/100	Chinese medicine vs. M (750 mg)	12 w	Feces	16S rRNA V3–V4 region	Post vs. pre	√	√	√		
Wu et al. (21), Europe	Newly T2D	22/18	M (425–1,700 mg) vs. placebo	4 m	Feces	DNA shotgun metagenomics	Post vs. pre		√	√		√
							With vs. without				√	√
Zhang et al. (7), China	Pre-diabetes	40/40	A (50–150 mg) vs. placebo	4 w	Feces	16S rRNA V3–V5 region	Post vs. pre	√	√	√		
Gu et al. (32), China	Newly T2D	51/43	A (75–450 mg) vs. G (5–15 mg)	3 m	Feces	DNA metagenomics	Post vs. pre	√		√		√
Su et al. (33), China	Prevalent T2D	59/36	A 150 mg vs. non-A	4 w	Feces	16S rDNA RT-qPCR	Post vs. pre			√		
Wang et al. (34), USA	Prevalent T2D	19/18	L vs. M (as usual)	18 w	Feces	16S rRNA V4 region	L vs. M	√	√	√		
							Post vs. pre		√	√		
Quasi-experimental studies (*n* = 5)												
Bryrup et al. (22), Denmark	Healthy	25	M (500–2,000 mg)	6 w	Feces	16S rRNA V4 region	Post vs. pre	√	√	√		
Huang et al. (23), Sweden	Prevalent T2D	23/7	M (as usual) vs. non-M	28 w	Feces	16S rRNA gene T-RFLP	With vs. without	√		√	√	
Sun et al. (24), China	Newly T2D	22	M (2,000 mg)	3 d	Feces	DNA metagenomics	Post vs. pre	√	√	√		√
Elbere et al. (25), Latvia	Healthy	18	M (1,700 mg)	7 d	Feces	16S rRNA V3 region	Post vs. pre	√	√	√		
Napolitano et al. (26), UK	Prevalent T2D	14/14	on-M (as usual) vs. off-M	NA	Feces	16S rRNA V1, V2, V3 regions	On vs. off		√	√		√
Cross-sectional studies (*n* = 5)												
Zhang et al. (27), China	Prevalent T2D	51/26	M (as usual) vs. non-treatment	–	Feces	16S rRNA V1–V2 region	With vs. without	√	√	√		
Barengolts et al. (28), USA	Prevalent T2D	25/16	M (as usual) vs. non-M	–	Feces	16S rRNA V3–V4 region	With vs. without	√	√	√		
De La Cuesta-Zuluaga et al. (29), Columbia	Prevalent T2D	14/14	M (as usual) vs. non-M	–	Feces	16S rRNA V4 region	With vs. without	√	√	√		
Forslund et al. (30), Denmark	Prevalent T2D	58/17	M (as usual) vs. non-M	–	Feces	16S rDNA shotgun metagenomics	With vs. without	√		√		
Karlsson et al. (31), Sweden	Prevalent T2D	20/33	M (as usual) vs. non-M	–	Feces	DNA metagenomics	With vs. without			√		

A, acarbose; G, glipizide; M, metformin; L, liraglutide; T-RFLP, terminal restriction fragment length polymorphism; RT-qPCR, Real-time quantitative polymerase chain reaction; m, months; w, weeks; d, days; –, no information; NA, not available; vs., versus.

TABLE 3 | Characteristics of included animal studies.

References, country	Animals	Models	*N*	Treatment and daily dose	Duration	Specimens	Analysis methods	Comparison	Outcomes				
									α-diversity	β-diversity	Taxonomic composition	SCFAs	Bile acids
Studies in mice (*n* = 30)													
Ryan et al. (37), Ireland	Male C57BL/6	HFD	14/14	M (300 mg/kg) vs. non-treatment	12 w	Ceca	16S rRNA V3–V4 region	With vs. without	√	√	√		
Ji et al. (38), China	Male C57BL/6J	HFD	5/5	M (300 mg/kg) vs. non-treatment	3 w	Feces	16S rRNA V4 region	With vs. without	√	√	√		
Adeshirlarijaney et al. (41), USA	Male C57BL/6	HFD	10/NA	M (300 mg/kg IP) vs. vehicle	10 w	Feces	16S rRNA V4 region	With vs. without	√	√	√		
Liao et al. (60), China	Male C57BL/6	HFD	NA	A (400 mg/kg) vs. Si (4 g/kg) vs. Sa (300 mg/kg) vs. L (200 μg/kg) vs. normal saline	4 w	Feces	16S rDNA V3–V4 region	With vs. without (Si)		√	√	√	
								With vs. without (A, Sa, L)		√			
Madsen et al. (65), Denmark	Male C57BL/6	HFD	15/15	L (0.4 mg/kg) vs. vehicle	28 d	Feces	16S rDNA V3–V4 region and metagenomics	Post vs. pre	√	√	√		
Wang et al. (45), Korea	Male C57BL/6J	HFD	16/8	M (100 mg/kg) vs. non-treatment	10 w	Feces	16S rRNA gene RT-qPCR	With vs. without		√	√		
Lee et al. (48), Korea	Male C57BL/6N	HFD	6/6	M (250 mg/kg) vs. non-treatment	16 w	Ceca	16S rRNA V4 region	With vs. without	√	√	√		
Zhou et al. (53), China	Male C57BL/6J	HFD	NA	M (100 mg/kg) vs. non-treatment	4 w	Feces	16S rRNA gene RT-qPCR	With vs. without			√		
Do et al. (63), Korea	Male C57BL/6J	HFD	9/10	Vo (1 mg/kg) vs. non-treatment	12 w	Feces	16S rRNA V1–V3 region	With vs. without			√		√
Lee and Ko (55), Korea	Female C57BL/6	ND, HFD	NA	M (300 mg/kg) vs. non-treatment	10 w	Feces	16S rRNA V1–V3 region	With vs. without	√	√	√		
Shin et al. (56), Korea	C57BL/6	ND, HFD	12/12	M (300 mg/kg) vs. non-treatment	6 w	Feces	16S rRNA gene RT-qPCR	With vs. without		√	√		
Dong et al. (39), USA	KC	Gene knockout with HFCD	8/8	M (5 mg/ml in drinking water) vs. non-treatment	2 m	Duodena, ilea, ceca	16S rRNA V4 region	With vs. without	√	√	√		
Brandt et al. (40), Germany	Female C57BL/6J	FFCD	6–8/6–8	M (300 mg/kg) vs. non-treatment	4 d	Proximal small intestine	16S rRNA V1–V2 region	With vs. without	√	√	√		
Baxter et al. (61), USA	Male C57BL/6	HSD, PPD	25/5	A (25, 400 mg/kg) vs. non-treatment	2 w	Feces	16S rRNA V4 region	With vs. without		√	√	√	
								Post vs. pre		√	√	√	
Kishida et al. (64), Japan	Male C57BL/6J	HFHSD	10/10	Mi 0.04% in diet vs. non-treatment	12 w	Feces	16S rRNA V3–V4 region	With vs. without	√	√	√		
Olivares et al. (74), Belgium	Male C57BL/6J	WD	9/9	Vi (0.6 mg/mL in drinking water) vs. non-treatment	8 w	Ceca	16S rRNA V5–V6 region	With vs. without	√	√	√	√	
Zheng et al. (44), China	Male C57BL/6J	HFD/STZ	48/8	M (75, 200 mg/kg) vs. normal saline	5 w	Feces	16S rRNA V3–V4 region	With vs. without		√	√		

(Continued)

TABLE 3 | Continued

References, country	Animals	Models	*N*	Treatment and daily dose	Duration	Specimens	Analysis methods	Comparison	Outcomes				
									α-diversity	β-diversity	Taxonomic composition	SCFAs	Bile acids
Wang et al. (70), China	Male ApoE$^{-/-}$	HFD ± STZ	20/20/20	L (0.4 mg/kg) vs. Sa (10 mg/kg) vs. non-treatment	8 w	Feces	16S rRNA V1–V3 region	With vs. without (L, Sa)	√	√	√		
Xue et al. (36), China	Female C57BL/6J	DHEA+HFD	10/10	M (1.9 g/kg) vs. normal saline	21 d	Feces	16S rDNA V3–V4 region	With vs. without		√	√		
Moreira et al. (68), Brazil	Male C57BL/6J and female *ob/ob*	ND, HFD, gene knockout	24–48/24–48	L (400 μg/kg) vs. normal saline	15 d	Feces	16S rRNA V3–V4 region	With vs. without	√		√		
Ma et al. (46), China	C57BL/6	ND	10/9	M (300 mg/kg) vs. normal saline	30 d	Feces	16S rRNA	With vs. without	√	√	√		
Xu et al. (62), China	Male ICR	ND	5/5/5	A (4 mg/kg) vs. Vo (0.008 mg/kg) vs. non-treatment	2 w	Intestine	16S rRNA V4 region	With vs. without			√	√	
Zhang et al. (35), China	BKSLeprdb (*db/db*)	Gene knockout	5/5	M (113.75 mg/kg) vs. non-treatment	11 w	Feces	16S rRNA V3–V4 region	With vs. without	√	√	√	√	
Lee et al. (76), USA	C57BLKS/J-leprdb/leprdb (*db/db*)	Gene knockout	12/12	D (60 mg/kg in diet) vs. non-treatment	8 w	Feces	16S rRNA V4 region	With vs. without	√	√	√		
Li et al. (75), China	Male ICR MafA-deficient	Gene knockout	8/8	D (1.0 mg/kg) vs. normal saline	6 w	Intestine and feces	16S rRNA V3–V4 region	With vs. without	√	√	√	√	
Li et al. (78), China	Female KKAy	Gene knockout	6/6	P vs. distilled water	NA	Feces	16S rDNA DGGE	With vs. without	√	√	√		
Wang et al. (79), China	KKAy	Gene knockout	7/6	R (2 mg/kg) vs. distilled water	8 w	Intestine	Cultivation	With vs. without			√		
Smith et al. (59), USA	Offsprings of female CByB6 mF1/J and male C3D2F1/J	ND	71/72	A (1,000 ppm with diet) vs. non-treatment	17–25 m	Feces	16S rRNA V4 region	With vs. without	√	√	√	√	
Salomäki-Myftari et al. (52), Finland	Offsprings of homozygous OE-NPY	Gene knockout	NA	M (300 mg/kg) vs. vehicle (for dams)	18 d	Feces	16S rRNA V4–V5 region	With vs. without		√	√		
Mishima et al. (77), Japan	Male C57BL/6	Adenine induced renal failure	8/8	C (10 mg/kg) vs. vehicle	2 w	Ceca	16S rRNA V1–V2 region	With vs. without		√	√	√	
Studies in rats (*n* = 17)													
Bauer et al. (43), Canada	Male SD	HFD	6/6	M (200 mg/kg) vs. normal saline	1 d	Lumina	16S rRNA V3 region	With vs. without	√	√	√		
Zhang et al. (54), China	Male W	HFD	10/10	M (200 mg/kg) vs. vehicle	8 w	Feces	16S rRNA V3 region	With vs. without	√	√	√		
Pyra et al. (57), Canada	Male SD	HFHSD	20/10	M (300 mg/kg) vs. non-treatment	7 w	Ceca	DNA gene RT-qPCR	With vs. without			√		
Dennison et al. (72), Canada	Female SD	HFHSD	11–13/11–13	Si (10 mg/kg) vs. non-treatment	12 w	Feces	16S rRNA gene RT-qPCR	With vs. without			√		
Liu et al. (47), China	Male W	HFD/STZ	10/10	M (200 mg/kg) vs. non-treatment	4 w	Colon	16S rRNA V3–V4 region	With vs. without	√	√	√		

(Continued)

TABLE 3 | Continued

References, country	Animals	Models	*N*	Treatment and daily dose	Duration	Specimens	Analysis methods	Comparison	Outcomes				
									α-diversity	β-diversity	Taxonomic composition	SCFAs	Bile acids
Xu M et al. (42), China	SD	HFHSD/STZ	10/10	M (1.8 g/kg) vs. non-M	4 w	Feces	16S rDNA gene qPCR	With vs. without			√		
Zhang et al. (69), China	Male SD	HFD/STZ	6/6	L (0.4 mg/kg) vs. normal saline	NA	Feces	16S rRNA V3–V4 region	With vs. without	√	√	√		
Zhang et al. (6), China	Male SD	HFD/STZ	12/6	Vi (0.01, 0.02 g/kg) vs. vehicle	12 w	Feces	16S rRNA V3–V4 region	With vs. without	√	√	√		
Yan et al. (73), China	Male SD	HFHC/STZ	10/10	Si (10 mg/kg) vs. non-treatment	12 w	Feces	16S rRNA V1–V3 region	With vs. without	√	√	√		
Yuan et al. (67), China	Male SD	STZ	6/6	L (0.6 mg/kg) vs. non-L	NA	Feces	16S rRNA V3 region	With vs. without			√		
Zhang et al. (5), China	Male ZDF	Gene knockout	8/8/8/8	A (32.27 mg/kg) vs. M (215.15 mg/kg) vs. Si (10.76 mg/kg) vs. normal saline	4 w	Feces	16S rRNA V3–V4 region	A vs. M A vs. Si	√	√	√		
								With vs. without (A)	√	√	√		
								With vs. without (M, Si)		√	√		
Shin et al. (49), Korea	Male OLETF	Gene knockout	7/7	M (100 mg/kg) vs. water	12 w	Feces	16S rRNA V1–V3 region	With vs. without	√	√	√		
Wang et al. (50), Korea	Male OLETF	Gene knockout	NA	M (100 mg/kg) vs. distilled water	12 w	Feces	16S rRNA V3 region	With vs. without		√	√	√	
Han et al. (51), Korea	Male OLETF	Gene knockout	7/7	M (100 mg/kg) vs. distilled water	12 w	Feces	16S rRNA V1–V2 region	With vs. without			√		√
Zhao et al. (58), China	Male GK	Gene knockout	6/6	A (50 mg/kg) vs. normal saline	8 w	Colon, feces	16S rRNA V3–V4 region	With vs. without	√	√	√		
Zhao et al. (66), China	Male W and GK	HFD, gene knockout	16/16	L (400 μg/kg) vs. normal saline	12 w	Feces	16S rRNA V3–V4 region	With vs. without	√	√	√		
Kaya et al. (71), Japan	Male OLETF	Gene knockout and PS	10/10	An (45 mg/kg) vs. vehicle	8 w	Feces	16S rRNA V4 region	With vs. without	√		√		

A, acarbose; An, anagliptin; C, canagliflozin; D, dapagliflozin; G, glipizide; Mi, miglitol; M, metformin; L, liraglutide; P, pioglitazone; R, rosiglitazone; Si, sitagliptin; Sa, saxagliptin; Vo, voglibose; Vi, vildagliptin; HFD, high-fat diet; ND, normal-chow diet; HFHSD, high-fat high-sucrose diet; HFHCD, high-fat high-carbohydrate diet; DHEA, trans-dehydroandrosterone; HFCD, high-fat high-calories diet; FFCD, fat-, fructose-, and cholesterol-rich diet; HSD, high-starch diet; PPD, plant plolysaccharide diet; WD, Western diet; STZ, streptozocin intraperitoneal injection; DHEA, trans-dehydroandrosterone; PS, pocrine serum intraperitoneal injection; IP, Intraperitoneal injection; SD rats, Sprague-Dawley rats; ZDF rats, Male Zucker diabetic fatty rats, induced by leptin receptor gene knockout; KC mice, LSL-KrasG12D/+ and p48-Cre+/- mice, induced by LSL-KRASG12D and Cre alleles knockout; OLETF rats, Otsuka Long-Evans Tokushima Fatty rats, induced by spontaneous CCK_1 receptor knockout; OE-NPY mice, homozygous transgenic OE-NPY mice, induced by transgenic mice overexpressing Neuropeptide Y under dopamine–β-hydroxylase promoter; W rats, Wistar rats; GK rats, Goto-Kakizaki rats, induced by polygenic Wistar substrain; ob/ob mice, mice model induced by Lep^{ob} gene knockout; ICR MafA-deficient mice, model induced by targeted disruption of the mafA gene in ICR mice; db/db mice, model induced by mutation in the leptin receptor gene in mice; KKAy mice, induced by transfer the yellow obese gene (A^y) into KK mice; RT-qPCR, Real-time quantitative polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; m, months; w, weeks; d, days; NA, not available; vs., versus.

TABLE 4 | Effects of anti-hyperglycemic drugs on specific taxa in human gut microbiota, categorized by the target research populations[a].

Specific taxa	Phylum	N[b]	N/N[c]	Healthy	Obese	Pre-diabetic	Newly T2D	Prevalent T2D
G_*Alistipes*	Bacteroidetes	2	151/151				↓ A (32), M (20)	
G_*Bacteroides*	Bacteroidetes	5	233/233		↔ M (19)	↓ A (7)	↓ A (32), M (20, 24)	
S_*Bacteroides dorei*	Bacteroidetes	2	73/73				↓ A (32), M (24)	
S_*Bacteroides finegoldii*	Bacteroidetes	2	73/73				↓ A (32), M (24)	
S_*Bacteroides intestinalis*	Bacteroidetes	2	73/73				↓ A (32), M (24)	
S_*Bacteroides stercoris*	Bacteroidetes	2	73/73				↓ A (32), M (24)	
S_*Bacteroides thetaiotaomicron*	Bacteroidetes	2	73/73				↓ A (32), M (24)	
S_*Bacteroides uniformis*	Bacteroidetes	2	73/73				↓ A (32), M (24)	
S_*Bacteroides vulgatus*	Bacteroidetes	2	73/73				↓ A (32), M (24)	
G_*Bifidobacterium*	Actinobacteria	2	73/73				↑ A (32), M (21)	
S_*Bifidobacterium adolescentis*	Actinobacteria	2	73/73				↑ A (32), M (21)	
G_*Clostridium*	Firmicutes	2	76/76	↓ M (22)			↓ A (32)	
S_*Clostridium leptum*	Firmicutes	2	71/84				↓ A (32)	↓ M (31)
F_*Lachnospiraceae*	Firmicutes	2	140/140			↓ A (7)	↑ M (20)	
G_*Lactobacillus*	Firmicutes	3	111/111		↔ M (19)	↑ A (7)	↑ A (32)	
S_*Lactobacillus gasseri*	Firmicutes	2	73/73				↑ A (32), M (21)	
G_*Megasphaera*	Firmicutes	2	54/54			↑ A (7)		↑ M (29)
S_*Pseudoflavonifractor capillosus*	Firmicutes	2	73/73				↓ A (32), M (21)	
S_*Ruminococcus* sp. *5_1_39BFAA*	Firmicutes	2	73/73				↓ A (32), ↑ M (21)	

[a]*The target research populations include obese, pre-diabetic, newly Type 2 diabetes (T2D), prevalent T2D;* [b]*Number of studies;* [c]*Number of participants (treatment/comparison); F, family; G, genus; S, species; M, metformin; A, acarbose; ↑, significant increase; ↓, significant decrease; ↔, no significant difference.*

This decrease was also noted in another two studies, in which liraglutide was given to in rat models induced by diets and gene knockout (66, 69).

Responses of the phylum *Bacteroidetes* to different anti-hyperglycemic agents were investigated in 15 rodent studies. No difference was noted in studies treated with acarbose (5, 58). Five out of the seven studies on the genus *Bacteroides* after using metformin (37, 42, 48, 54, 56) revealed increased abundance of this genus in mice and rats.

Among 15 rodent studies (5, 6, 36, 37, 41, 47, 48, 54, 56, 58, 66, 69, 70, 73, 75) on the phylum *Firmicutes,* the results were inconclusive among those treated with metformin (5, 36, 37, 41, 47, 48, 54, 56), acarbose (5, 58), liraglutide (66, 69, 70), and sitagliptin (5, 73). The genus *Lactobacillus* was the focus of 13 studies (5, 35, 41–43, 47, 53, 54, 58, 62, 66, 70, 73). Six out of the eight studies treated with metformin (5, 35, 43, 53, 54, 62) saw an increase in this genus in mice and rats, while the results in studies treated with acarbose (5, 58, 62), liraglutide (66, 70), and sitagliptin (5, 73) were inconclusive.

With respect to other phyla, there was a trend of decrease in the phylum *Proteobacteria* in mice treated with metformin and liraglutide, while *Verrucomicrobia* and *Tenericutes* increased after treated with metformin and liraglutide, respectively. However, results for the phyla *Actinobacteria*, *Cyanobacteria, Elusimicrobia*, and *Fusobacteria* were conflicting. The genus *Akkermanisa* (phylum *Verrucomicrobia*) increased in eight studies treated with metformin using dietary or genetic models (35, 37, 38, 41, 44, 48, 54, 56). Three of the four studies with metformin (41, 53, 55) reported an increase in the species *A. muciniphila*, and another two studies reported a similar increase in this species after treating with liraglutide (65, 68).

Diversity

Ten human studies treated with metformin (19, 20, 22–25, 27–30) and two studies treated with acarbose (7, 32) have provided the results of α-diversity. The results from those metformin studies, however, were conflicting, while both acarbose studies reported a decrease in the α-diversity among pre-diabetic and T2D patients. β-diversity was assessed in ten studies treated with metformin (19–22, 24, 25, 27–29, 34), of which six studies (20–22, 24, 27, 34) revealed a significant difference after the treatment in healthy participants and T2D patients. This difference was also noted in pre-diabetic patients treated with acarbose (32), and T2D patients treated with liraglutide (34) (**Table 8**).

Similar results with metformin and acarbose were reported in mouse studies. The effects of metformin on α-diversity were conflicting across different models, while the α-diversity decreased consistently in three studies treated with acarbose (5, 58, 59). Moreover, the results were inconsistent among those studies treated with liraglutide, sitagliptin, vildagliptin, and saxagliptin. In terms of β-diversity, there was higher cumulative evidence of significant difference after using metformin (5, 36–39, 41, 43–50, 54–56), and similar results were consistently reported among those studies treated with acarbose (5, 58–61), liraglutide (60, 65, 66, 69, 70), sitagliptin (5, 60, 73), and

TABLE 5 | Consistent and inconsistent effects of each anti-hyperglycemic drug on specific taxa in human gut microbiota, categorized by the target research populations[a].

Drug/specific taxa	Phylum	*N*[b]	*N*/*N*[c]	Healthy	Obese	Pre-diabetic	Newly T2D	Prevalent T2D
METFORMIN								
Consistent results								
G_*Fusobacterium*	Fusobacteria	2	151/126				↑ (20, 27)	
S_*Akkermansia muciniphila*	Verrucomicrobia	2	36/36				↑ (21)	↑ (29)
G_*Escherichia*	Proteobacteria	6	243/202	↑ (22, 25)	↑ (19)		↑ (20, 21)	↑ (30)
G_*Shigella*	Proteobacteria	4	163/163	↑ (22, 25)	↑ (19)		↑ (20)	
S_*Citrobacter koseri*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Citrobacter rodentium*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Enterobacter cloacae*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Escherichia coli*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Klebsiella pneumonia*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Erwinia amylovora*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Pectobacterium wasabiae*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Dickeya dadantii*	Proteobacteria	2	42/55				↑ (21)	↑ (31)
S_*Clostridium bartlettii*	Firmicutes	2	42/55				↓ (21)	↓ (31)
S_*Clostridium botulinum*	Firmicutes	2	42/55				↓ (21)	↓ (31)
Inconsitent results								
G_*Bacteroides*	Bacteroidetes	3	142/142		↔ (19)		↓ (20, 24)	
G_*Prevotella*	Bacteroidetes	2	34/34		↔ (19)			↑ (29)
G_*Blautia*	Firmicutes	3	138/138	↔ (25)	↔ (19)		↑ (20)	
G_*Intestinibacter*	Firmicutes	4	125/84	↓ (22)	↔ (19)		↓ (21)	↓ (30)
F_*Enterobacteriaceae*	Proteobacteria	2	69/44	↑ (25)				↓ (27)
Acarbose								
Consistent results								
G_*Bacteroides*	Bacteroidetes	2	91/91			↓ (7)	↓ (32)	
G_*Lactobacillus*	Firmicutes	2	91/91			↑ (7)	↑ (32)	
S_*Bidifobacterium longum*	Actinobacteria	2	110/110				↑ (32)	↑ (33)

[a]*The target research populations include obese, pre-diabetic, newly Type 2 diabetes (T2D), prevalent T2D;* [b]*Number of studies;* [c]*Number of participants (treatment/comparison); F, family; G, genus; S, species; ↑, significant increase; ↓, significant decrease; ↔, no significant difference.*

vildagliptin (6, 74) across different mouse models. Evidence for the effects of other drugs was limited (**Table 9**).

Short-Chain Fatty Acids (SCFAs)

In human studies, changes in the levels of three main SCFAs (acetate, propionate, and butyrate) in feces and sera were reported in three studies treated with metformin (19, 21, 23) (**Supplementary Table 3**). Wu et al. (21) found that the levels of fecal butyrate and propionate increased in T2D male patients. However, no difference in fecal levels of these two SCFAs was noted among obese women in Ejtahed's study (19). In contrast, fecal acetate levels decreased in obese women (19) did not change among T2D patients in Wu's study (21). Huang et al. (23) reported that the serum levels of all three SCFAs remained unchanged after treating with metformin in T2D patients.

In mouse studies, fecal levels of SCFAs after metformin interventions were assessed in *db*/*db* mice (35) and OLETF rats (50) (**Supplementary Table 3**). It was found that levels of acetate and butyrate increased, but propionate levels remained unchanged. The effects of acarbose on these SCFAs levels were also assessed in dietary models (59, 61, 62). These studies showed consistent results of increased levels of butyrate in feces and ceca, but the levels of acetate and propionate varied in a diet-dependent manner.

Bile Acids

In human studies, three clinical trials treated with metformin (21, 24, 26) and one randomized trial with acarbose and glipizide (32) were carried out in T2D patients to assess the respective effects on the fecal and serum levels of bile acids (**Supplementary Table 4**). Regarding metformin, one study (24) showed increases in the fecal level of conjugated secondary bile acids, while no difference was reported in the other two studies (21, 26). Two of these three studies (21, 24) reported increases in the blood level of

TABLE 6 | Effects of anti-hyperglycemic drugs on gut microbiota in mouse models, categorized by the treated, individual anti-hyperglycemic drug.

Specific taxon	N^a	M	A	Mi	Vo	L	An	Sa	Si	Vi	C	D	R
Firmicutes/bacteroidetes	9	↓ (2)	↔		↓	↓ (2)	↓			↓		↓	
Phylum *Bacteroidetes*													
P_*Bacteriodetes*	15	↔(3), ↓, ↑ (5)	↔(2)			↓, ↑ (2)		↓	↔, ↑ (2)	↑		↑	
C_*Bacteroidia*	3	↑				↓, ↑							
F_*Bacteroidaceae*	5	↑	↑			↓			↑	↑			
G_*Bacteroides*	18	↔(2), ↓, ↑ (5)	↑		↑	↔, ↑, ↓	↑	↓	↑ (2)	↔, ↑		↑	
S_*Bacteroides acidifaciens*	2					↑				↑		↑	
G_*Butyricimonas*	3	↑ (2)				↑							
F_*Porphyromonadaceae*	4	↑ (2)				↓, ↑							
G_*Odoribacter*	4	↔, ↓ (2)	↔										
G_*Parabacteroides*	8	↓, ↑ (4)	↔			↓			↑				
F_*Prevotellaceae*	5	↑ (2)							↔,↑	↓			
G_*Prevotella*	8	↓, ↑ (3)				↓	↓	↓		↔			
G_*Prevotella_9*	3					↓, ↑				↑			
F_*Rikenellaceae*	3	↑ (2)		↑									
G_*Alistipes*	3	↑	↑		↑							↑	
F_*S24-7*	3		↓, ↑						↑				
Phylum *Firmicutes*													
P_*Firmicutes*	15	↔(4), ↓ (3), ↑ (2)	↔, ↑			↔, ↓, ↑		↑	↓, ↑	↓		↑	
G_*Turicibacter*	5	↔, ↓, ↑	↔			↑		↑					
O_*Lactobacillales*	2	↓				↑							
G_*Enterococcus*	7	↔, ↓ (3), ↑										↓, ↑	
F_*Lactobacillaceae*	5	↓, ↑	↓ (2)			↑							
G_*Lactobacillus*	13	↓ (2), ↑ (6)	↔, ↓, ↑			↓, ↑		↑	↓, ↑				
S_*Lactobacillus intesinalis*	1	↑	↑						↑				
S_*Lactobacillus johnsonii*	2	↑							↑	↔			
F_*Streptococcaceae*	5	↓ (2)				↓			↓	↑			
G_*Streptococcus*	4	↓	↔							↑			↓
C_*Clostridia*	2	↓				↓							
O_*Clostridiales*	2	↓				↓							
F_*Clostridiales_vadinBB60_g*	2					↑			↓				
F_*Christensenellaceae*	3	↑				↓				↓			
G_*Christensenellaceae R_7_g*	3		↔			↔, ↓				↓			
G_*Candidatus Arthromitus*	3	↔	↔			↓							
G_*Clostridium*	3	↓				↑			↔				
G_*Ruminiclostridium*	2		↔						↓				
G_*Ruminiclostridium 6*	2		↓		↓	↓				↓			
G_*Ruminiclostridium 9*	3		↔			↔			↓				
F_*Ruminococcaceae*	6	↑	↓			↔, ↑			↓	↓			
G_*Ruminococcaceae_UCG_005*	1		↓		↓								
G_*Ruminococcus*	5	↓ (2), ↑ (2)							↑				
G_*Ruminococcus 2*	2		↓, ↑		↑								
F_*Lachnospiraceae*	5	↓	↔			↔, ↓			↓				
G_*Blautia*	8	↓, ↑	↑		↑	↑ (2)			↓ (2)			↑	
G_*Lachnoclostridium*	3	↓	↔			↑							
G_*Lachnospiraceae_nk4a136_g*	2					↔						↑	
G_*Marvinbryantia*	2		↔			↓							

(Continued)

TABLE 6 | Continued

Specific taxon	N[a]	M	A	Mi	Vo	L	An	Sa	Si	Vi	C	D	R
G_*Roseburia*	7	↔, ↓	↔			↔, ↓			↔, ↓				
G_*Peptococcus*	2	↓	↔										
G_*Romboustia*	2	↓				↓							
G_*Anaerotruncus*	6	↔, ↓	↔			↓			↓	↓			
G_*Flavonifractor*	2	↓				↑							
G_*Oscillospira*	6	↓, ↑				↑				↓	↓	↓	
F_*Dehalobacteriaceae*	3	↓, ↑		↑									
F_*Erysipelotrichaceae*	5	↑	↔, ↑	↓		↑							
O_*Erysipelotrichales*	2	↑				↑							
Phylum *Actinobacteria*													
P_*Actinobacteria*	5	↔	↑			↔, ↓					↓		
F_*Bifidobacteriaceae*	2	↓	↑										
G_*Bifidobacterium*	8	↔(2), ↑, ↓	↑			↑			↑		↓		
S_*Bifidobacterium* spp.	2	↓								↔			
G_*Corynebacterium 1*	2		↔						↑				
G_*Enterorhabdus*	2		↔			↓							
Phylum *Cyanobacteria*													
P_*Cyanobacteria*	2	↓	↓			↑			↓				
Phylum *Elusimicrobia*													
P_*Elusimicrobia*	2					↔				↓			
G_*Allobaculum*	10	↔, ↓ (2), ↑ (2)	↑			↔, ↑		↑ (2)					
Phylum *Fusobacteria*													
P_*Fusobacteria*	3	↓, ↑				↔							
Phylum *Proteobacteria*													
P_*Proteobacteria*	8	↓ (3), ↑	↓			↔, ↓ (2)			↓↑			↑	
C_*Alphaproteobacteria*	2	↓								↑			
G_*Desulfovibrio*	5	↔, ↓	↓		↓	↑			↓				
F_*Desulfovibrionaceae*	4	↓	↓	↑					↓				
F_*Enterobacteriacae*	2	↑										↓	
G_*Escherichia*	3	↔, ↑											↓
G_*Helicobacter*	2	↓				↓							
Phylum *Tenericutes*													
P_*Tenericutes*	6	↔, ↑	↔			↑ (2)			↔, ↑	↓			
C_*Mollicutes*	2	↑				↓							
Phylum *Verrucomicrobia*													
P_*Verrucomicrobia*	8	↔, ↓ (2), ↑ (5)	↓			↑							
F_*Verrucomicrobiaceae*	5	↑ (4)	↓ (2)										
G_*Akkermansia*	9	↑ (8)				↑							
S_*Akkermansia muciniphila*	6	↔, ↑ (3)				↑ (2)							

[a]*Number of studies; M, metformin; A, acarbose; Mi, miglitol; Vo, voglibose; L, liraglutide; An, anagliptin; Sa, saxagliptin; Si, sitagliptin; Vi, vildagliptin; C, canagliflozin; D, dapagliflozin; R, rosiglitazone; P, Phylum; C, Class; O, Order; F, Family; G, Genus; S, Species; ↑, significant increase; ↓, significant decrease; ↔, no significant difference; (n), Number of studies (≥2) reported the same results.*

secondary bile acids, while the other one (26) revealed an inverse trend. Concerning changes in total and primary bile acids, their levels in feces were unchanged among these three trials, but results in blood levels were conflicting (21, 26). The random trial assessing the effects of acarbose and glipizide on bile acid levels in newly diagnosed T2D patients (32) showed that acarbose might increase the plasma and fecal levels of primary bile acids, accompanied by decreases in secondary bile acids. In contrast, no significant changes in bile acid levels were found in patients treated with glipizide.

TABLE 7 | Consistent and inconsistent effects of each anti-hyperglycemic drug on specific taxa in mouse gut microbiota, categorized by mice or rat models with three distinct animal models.

Specific taxa	Phylum	*N*[a]	Trend[b]	Mice models			Rat models		
				Normal	Dietary or STZ	Gene knockout	Normal	Dietary or STZ	Gene knockout
METFORMIN									
Consistent results									
Firmicutes/Bacteroidetes		2	↓		↓ (2)				
F_*Porphyromonadaceae*	Bacteroidetes	2	↑	↑	↑				
F_*Prevotellaceae*	Bacteroidetes	2	↑	↑				↑	
F_*Rikenellaceae*	Bacteroidetes	2	↑	↑ (2)					
G_*Butyricimonas*	Bacteroidetes	2	↑		↑	↑			
F_*Enterococcaceae*	Firmicutes	2	↓		↓			↓	
F_*Streptococcaceae*	Firmicutes	2	↓		↓			↓	
F_*Verrucomicrobiaceae*	Verrucomicrobia	4	↑	↑ (2)	↑ (3)				
G_*Akkermansia*	Verrucomicrobia	8	↑		↑ (6)	↑		↑	
S_*Akkermansia* spp.	Verrucomicrobia	2	↑	↑					↑
F_*Alcaligenaceae*	Proteobacteria	2	↑		↑			↑	
Inconsistent results									
P_*Bacteroidetes*	Bacteroidetes	8	↑	↔	↓, ↑ (4)			↔, ↑	↔
G_*Bacteroides*	Bacteroidetes	7	↑	↑	↔, ↓, ↑ (2)	↔		↑ (2)	
G_*Odoribacter*	Bacteroidetes	3	↓		↓	↔			↓
G_*Parabacteroides*	Bacteroidetes	5	↑	↓	↓, ↑ (4)				
G_*Prevotella*	Bacteroidetes	4	↑		↑	↓		↑ (2)	
P_*Firmicutes*	Firmicutes	8	○	↔	↔, ↑, ↓ (3)			↔(2)	↑
G_*Allobaculum*	Firmicutes	5	○	↓	↑, ↓	↔		↑	
G_*Anaerotruncus*	Firmicutes	2	○		↓	↔			
G_*Blautia*	Firmicutes	2	○		↓			↑	
G_*Christensenella*	Firmicutes	2	○		↑	↔			
G_*Coprococcus*	Firmicutes	3	↓		↓ (2)	↑			
F_*Dehalobacteriaceae*	Firmicutes	2	○		↓				↑
G_*Dehalobacterium*	Firmicutes	2	○		↓				↑
G_*Enterococcus*	Firmicutes	5	↓		↓ (2)			↔, ↑, ↓	
F_*Lactobacillaceae*	Firmicutes	2	○					↑, ↓	
G_*Lactobacillus*	Firmicutes	8	↑		↑, ↓	↑		↓, ↑ (3)	↑
G_*Lactococcus*	Firmicutes	2	○		↑, ↓				
G_*Oscillospira*	Firmicutes	2	○		↑, ↓				
G_*Roseburia*	Firmicutes	2	○			↔		↓	
G_*Ruminococcus*	Firmicutes	4	○	↑	↓ (2)	↑			
G_*Turicibacter*	Firmicutes	3	○	↑		↔, ↓			
G_*Bifidobacterium*	Actinobacteria	4	○		↔	↔		↑, ↓	
P_*Fusobacteria*	Fusobacteria	2	○		↑				↓
C_*Fusobacteriia*	Fusobacteria	2	○		↑				↓
O_*Fusobacteriales*	Fusobacteria	2	○		↑				↓
P_*Proteobacteria*	Proteobacteria	4	↓		↓			↑, ↓	↓
G_*Desulfovibrio*	Proteobacteria	2	○		↓	↔			
G_*Escherichia*	Proteobacteria	2	○		↑			↔	
S_*Escherichia coli*	Proteobacteria	2	○		↓			↔	
G_*Klebsiella*	Proteobacteria	2	○					↔, ↑	

(Continued)

TABLE 7 | Continued

Specific taxa	Phylum	N[a]	Trend[b]	Mice models			Rat models		
				Normal	Dietary or STZ	Gene knockout	Normal	Dietary or STZ	Gene knockout
G_*Parasutterella*	Proteobacteria	3	↑	↑	↔			↑	
G_*Proteus*	Proteobacteria	2	○		↑	↓			
G_*Sutterella*	Proteobacteria	3	↑		↑	↔		↑	
G_*Trabulsiella*	Proteobacteria	2	○		↓	↔			
P_*Tenericutes*	Tenericutes	2	○		↑				↔
P_*Verrucomicrobia*	Verrucomicrobia	7	↑	↔	↑ (4)			↑, ↓	↓
S_*Akkermansia muciniphila*	Verrucomicrobia	4	↑		↔, ↑ (3)				
G_*AF12*	NA	2	○		↓	↔			
ACARBOSE									
Consistent result									
F_*Lactobacillaceae*	Firmicutes	2	↓	↓	↓				
Inconsistent results									
F_*S24-7*	Bacteroidetes	2	○	↑	↓				
P_*Firmicutes*	Firmicutes	2	○			↔			↑
F_*Erysipelotrichaceae*	Firmicutes	2	○	↔	↑				
G_*Lachnospiraceae UCG-001*	Firmicutes	2	○	↔					↑
G_*Lactobacillus*	Firmicutes	3	○	↔		↑			↓
G_*Ruminococcus 2*	Firmicutes	2	○	↓					↑
DAPAGLIFLOZIN									
Inconsistent result									
G_*Enterococcus*	Firmicutes	2	○			↑, ↓			
LIRAGLUTIDE									
Consistent results									
Firmicutes/Bacteroidetes		2	↓				↓	↓	↓
G_*Blautia*	Firmicutes	2	↑		↓				↓
S_*Akkermansia muciniphila*	Verrucomicrobia	2	↑		↑ (2)				
Inconsistent results									
P_*Actinobacteria*	Actinobacteria	2	○		↓			↔	↔
P_*Bacteroidetes*	Bacteroidetes	3	↑		↓			↑ (2)	↑
G_*Bacteroides*	Bacteroidetes	3	○		↓			↑	↔
C_*Bacteroidia*	Bacteroidetes	2	○		↓			↑	↑
O_*Bacteroidales*	Bacteroidetes	2	○		↓			↑	
F_*Porphyromonadaceae*	Bacteroidetes	2	○		↓			↑	
G_*Prevotella_9*	Bacteroidetes	2	○					↓, ↑	↑
P_*Firmicutes*	Firmicutes	3	○		↑			↔, ↓	↔
G_*Allobaculum*	Firmicutes	2	○		↑				↔
G_*Christensenellaceae _R_7_group*	Firmicutes	2	○					↔, ↓	↔
F_*Lachnospiraceae*	Firmicutes	2	○		↓			↔	↔
F_*Ruminococcaceae*	Firmicutes	2	○					↔, ↑	↔
G_*Lactobacillus*	Firmicutes	2	○		↑			↓	↔
G_*Roseburia*	Firmicutes	2	○		↓				↔
P_*Proteobacteria*	Proteobacteria	3	↓	↓	↓ (2)	↓		↔	↔
P_*Tenericutes*	Tenericutes	2	○					↔, ↓	↔
P_*Verrucomiacrobia*	Verrucomiacrobia	2	○	↑	↑			↔	↔

(Continued)

TABLE 7 | Continued

Specific taxa	Phylum	N[a]	Trend[b]	Mice models			Rat models		
				Normal	Dietary or STZ	Gene knockout	Normal	Dietary or STZ	Gene knockout
SITAGLIPTIN									
Consistent results									
G_*Bacteroides*	Bacteroidetes	2	↑		↑			↑	
G_*Blautia*	Firmicutes	2	↓		↓			↓	
Inconsistent results									
P_*Bacteroidetes*	Bacteroidetes	2	○		↑				↔
F_*Prevotellaceae*	Bacteroidetes	2	○		↑			↔	
P_*Firmicutes*	Firmicutes	2	○					↓	↑
G_*Lactobacillus*	Firmicutes	2	○					↓	↑
G_*Roseburia*	Firmicutes	2	○		↓			↔	
P_*Proteobacteria*	Proteobacteria	2	○					↑	↓
P_*Tenericutes*	Tenericutes	2	○					↑	↔
VILDAGLIPTIN									
Consistent result									
G_*Oscillibacter*	Firmicutes	2	↓		↓				
Inconsistent result									
G_*Bacteroides*	Bacteroidetes	2	○		↔			↑	

P, Phylum; C, Class; O, Order; F, Family; G, Genus; [a]Number of studies; [b]Trend of alteration (reported in > 50% of studies): ↑, a trend of increase; ↓, a trend of decrease; ○, inconclusive results; Alteration of specific taxa: ↑, significant increase; ↓, significant decrease; ↔, no significant difference; (n), number of papers (≥2) reported the same results.

TABLE 8 | Effects of anti-hyperglycemic drug on diversity of human gut microbiota.

Drugs	Object	α-diversity		β-diversity[c]	References
		Richness[a]	Evenness[b]		
Metformin	Healthy	–	↓	ns	(25)
		ns	ns	≠	(22)
	Obese	ns	ns	ns	(19)
	Newly T2D	ns	↑	≠	(20)
		–	↓	≠	(24)
	Prevalent T2D	–	–	≠	(21)
		ns	ns	–	(23)
		–	↓	≠	(27)
		–	ns	ns	(28)
		ns	–	ns	(29)
		ns	–	–	(30)
		–	–	≠	(34)
Acarbose	Pre-diabetic	ns	↓	≠	(7)
	Newly T2D	↓	↓	–	(32)
Liraglutide	Prevalent T2D	–	–	≠	(34)
Glipizide	Newly T2D	ns	ns	–	(32)

[a]Richness was assessed by Chao1, ACE, and Rarefaction indices, gene count, number of OTUs, or number of species; [b]Evenness was assessed by Shannon, Simpson indices; [c]β-diversity was assessed by UniFrac (weighted, unweighted), Bray-Curtis, Jensen-Shannon, or Jaccard distances using Principal Component Analysis (PCA) and Principal Coordinates Analysis (PCoA); ↑, significant increase; ↓, significant decrease; ≠ , significant difference; ns, no significant difference; –, no information.

TABLE 9 | Effects of anti-hyperglycemia drugs on diversity in mouse gut microbiota.

Drugs	Objects	Models	α-diversity		β-diversity[c]	References
			Richness[a]	Evenness[b]		
Metformin	Mice	ND	–	ns	≠	(46)
			ns	–	ns	(55)
			–	ns	ns	(40)
			–	–	ns	(56)
		HFD	–	↓	≠	(37)
			–	ns	≠	(38)
			ns	–	≠	(41)
			–	–	≠	(45)
			ns	ns	≠	(48)
			↓	–	≠	(55)
					≠	(56)
		HFCD	ns	ns	≠	(39)
		FFCD	–	ns	ns	(40)
		HFD/STZ	–	–	≠	(44)
		DHEA/HFD	–	–	≠	(36)
		db/db	ns	↑	ns	(35)
		OE-NPY	–	–	ns	(52)
	Rats	HFD	ns	ns	≠	(43)
		HFD/STZ	↓	↓	≠	(54)
			↑	ns	≠	(47)
		OLETE	↑	–	≠	(49)
			–	–	≠	(50)
		ZDF	–	–	≠	(5)
Acarbose	Mice	ND	↓	↓	≠	(59)
		HSD	–	–	≠	(61)
		HFD	–	–	≠	(60)
	Rats	GK	–	↓	≠	(58)
		ZDF	↓	↓	≠	(5)
Miglitol	Mice	HFHSD	–	ns	≠	(64)
Liraglutide	Mice	ND	↓	–	–	(68)
		HFD	ns	ns	≠	(65)
			↑	–	–	(68)
			ns	↓	≠	(70)
			–	–	≠	(60)
		HFD/STZ	ns	ns	≠	(70)
		ob/ob	↑	–	–	(68)
	Rats	HFD/STZ	↓	↓	≠	(69)
		GK	ns	ns	≠	(66)
		W	↓	↓	≠	(66)
Sitagliptin	Mice	HFD	–	–	≠	(60)
	Rats	HFHC/STZ	↑	↑	≠	(73)
		ZDF	–	–	≠	(5)
Vildagliptin	Mice	WD	ns	ns	≠	(74)
		HFD/STZ	↓	↓	≠	(6)
Saxagliptin	Mice	HFD	–	–	≠	(60)
			ns	↓	ns	(70)

(Continued)

TABLE 9 | Continued

Drugs	Objects	Models	α-diversity		β-diversity[c]	References
			Richness[a]	Evenness[b]		
		HFD/STZ	ns	ns	ns	(70)
Anagliptin	Rats	OLETF & PS	ns	ns	–	(71)
Dapagliflozin	Mice	MafA-deficient	↓	↓	ns	(75)
	Mice	*db/db*	ns	ns	ns	(76)
Canagliflozin	Mice	Adenine	–	–	≠	(77)
Pioglitazone	Mice	KKAy	–	↓	≠	(78)

[a]*Richness was assessed by Chao1, ACE, and Rarefaction indices, gene count, number of OTUs, or number of species;* [b]*Evenness was assessed by Shannon, Simpson indices;* [c]*β-diversity was assessed by UniFrac (weighted, unweighted), Bray-Curtis, Jensen-Shannon, or Jaccard distances using Principle Component Analysis (PCA) and Principle Coordinates Analysis (PCoA); ↑, significant increase; ↓, significant decrease; ≠ , significant difference; ns, no significant difference; –, no information.*

As for rodent studies, one study in rats (51) revealed that the fecal level of total bile acids increased while the levels in liver tissues were decreased after metformin intervention. One study in mice (63) found that voglibose treatment was associated with increases in serum levels of primary bile acids, accompanied by decreases in serum levels of secondary bile acids (**Supplementary Table 4**).

Associations With Host Metabolic Parameters

Among pre-diabetic and T2D patients treated with metformin (20, 21) or acarbose (7, 32, 33), alterations in certain specific taxa in human gut microbiota were associated with improvement in HbA1C and fasting blood glucose values, body weights, and lipid profiles (**Table 10**). For instance, increments in the genera *Escherichia, Shigella, Subdoligranulum,* and *Dialister*, and the species *Bifidobacterium adolescentis, Bifidobacterium longum*, and *Lactobacillus gasseri* were inversely associated with HbA1C after treating with metformin or acarbose (7, 20, 21, 32). In addition, there were inverse associations between increases in the genus *Blautia* and fasting blood glucose after treating with metformin (20).

Mouse studies treated with metformin (44, 50, 55, 57), liraglutide (65, 66, 70), and saxagliptin (70) also explored the relationship between changes in the compositions of gut microbiota and improvement in various metabolic parameters (**Table 11**). It was found that some related specific taxa after treating with metformin in mice (i.e., *Bacteroides* spp., *Blautia*) were different from that in humans.

DISCUSSION

Our study provides a comprehensive review to report human and animal data separately about reciprocal interactions between anti-hyperglycemic drugs and specific taxonomic groups of gut microbiota. While other reviews suggest the effects of anti-hyperglycemic drugs on gut microbiota without discerning findings from either human or animal studies (8, 11, 13), this systematic review attempts to fill the gap of these reviews to try to explore the associations among anti-hyperglycemic agents, specific taxonomic patterns of gut microbiota, and glucose control or metabolic profiles mainly in humans, as compared to those reported in mouse studies. Further, the fact that three-quarters of included studies were published in and after 2017 implies a growing interest in this clinical question for an up-to-date systematic review.

Of the 17 human studies selected, the majority of these studies focus on either newly diagnosed or prevalent T2D patients, and directed toward investigating the interplay of metformin, and to a lesser extent, acarbose, with gut microbiota. Our results suggest that these two drugs mediate their glucose-lowering effect, in part, by stimulating beneficial gut bacteria that could produce metabolites to promote intestinal homeostasis (3, 9). We rationalize that alterations in gut microbiota compositions might also underlie the gastrointestinal side effects known to metformin, i.e., diarrhea and fecal incontinence (10, 25, 80). In contrast, results from other anti-hyperglycemic drugs analyzed in this study showed inconsistency with respect to their effects on the compositions of gut microbiota, which might be attributable to small numbers of studies and, equally important, differences in animal models and experimental conditions used among these studies.

Further, treatment durations of anti-hyperglycemic drugs in available studies, regardless of human or animal, varied to a great extent (i.e., few days to few months). Thus, the reported drug effects on the gut microbiota structure were diverse. Indeed, the anti-hyperglycemic drugs, i.e., metformin, could affect the intestinal bacterial compositions after 1 or several days of treatment (24, 25, 40, 43), or after prolonged periods of treatments (21). For instance, Wu et al. found that gut microbiota compositions after a 2- and 4-month treatment of metformin in newly T2D patients were not identical (21). In contrast, Wang et al. did not find significant changes in the gut microbiota compositions among T2D patients after different periods of metformin or liraglutide treatment, given their baseline gut microbiota compositions were unknown (34). Thus, there are no consistent findings on gut microbiota after various treatment durations of anti-hyperglycemic drugs, and further studies are warranted to explore the treatment duration of anti-hyperglycemics required for emergence of beneficial gut bacteria.

TABLE 10 | Association between specific taxa and human metabolic parameters.

Parameters	Association	Specific taxa	Alteration	Drugs	Participants	References
HbA1C	Negative	G_*Escherichia* G_*Shigella*	↑	M	Newly T2D	(20)
		S_ *Bifidobacterium adolescentis*	↑	M	Newly T2D	(21)
		S_*Lactobacillus gasseri* S_*Bifidobacterium longum*	↑	A	Newly T2D	(32)
		G_*Subdoligranulum* G_*Dialister*	↑	A	Pre-diabetic	(7)
Fasting blood glucose	Negative	G_*Blautia*	↑	M	Newly T2D	(20)
Body weight	Positive	S_*Bacteroides plebeius* S_*Bacteroides dorei* S_*Bacteroides vulgatus* S_*Clostridium bolteae*	↓	A	Newly T2D	(32)
	Negative	S_*Lactobacillus gasseri* S_*Bifidobacterium longum*	↑	A	Newly T2D	(32)
HDL cholesterol	Positive	S_*Bidifobacterium longum*	↑	A	Prevalent T2D	(33)
LDL cholesterol	Negative	G_*Blautia*	↑	M	Newly T2D	(20)

G, genus; S, species; M, metformin; A, acarbose; ↑, significant increase; ↓, significant decrease.

Evidence indicates that the use of metformin or acarbose in T2D patients was associated with increases in the abundance of beneficial bacteria, including the genera *Bifidobacterium* (phylum *Actinobacteria*) and *Lactobacillus* (phylum *Firmicutes*), and the species *A. muciniphila* (phylum *Verrucomicrobia*). The increase in the genus *Bifidobacterium* was positively associated with diabetes control, which is consistent with that reported in the review by Gurung et al. (1). In addition, two included studies showed an increase in two specific species of the genus *Bifidobacterium* (*B. adolescentis* and *B. longum*), which was inversely associated with HbA1C levels or body weights, and positively associated with HDL cholesterol levels among newly diagnosed T2D patients (21, 32).

A number of human studies have reported positive associations between the abundance of the genus *Lactobacillus* (phylum *Firmicutes*) and improved T2D control (81, 82). For example, T2D patients treated with acarbose showed increased *L. gasseri* levels, accompanied by lower HbA1C and body weights (32). In addition, as several species in the genus *Lactobacillus* have been used as probiotics, administration of these *Lactobacillus* strains showed beneficial effects on glycemic control and lipid profiles in T2D patients (4). Moreover, almost all animal studies that tested the efficacy of several species from the genus as probiotics for T2D reported improvements of glucose parameters (1).

A previous report found decreased abundance in the mucin-degrading bacterium *A. muciniphila* in patients with metabolic disorders, including obesity, impaired glucose tolerance, and diabetes, which were associated with insulin resistance, dyslipidemia, and overweight (83). Two other studies showed increased amounts of *A. muciniphila* in newly diagnosed and prevalent T2D patients treated with metformin, which, however, did not provide pertinent information on metabolic parameters. The potential role of this mucin-degrading bacterium in ameliorating metabolic disorders was further confirmed by a series of animal experiments. For example, mice treated with metformin and liraglutide showed increased levels of *A. muciniphila* in association with improved control of blood glucose and body weight (55, 65). More importantly, HFD-fed mice treated with *A. muciniphila* exhibited similar improvements in glucose tolerance and goblet cell production and inflammatory regulations as compared to the metformin treatment group (56).

The effect of metformin and acarbose on the abundance of different species of the genus *Bacteroides* (phylum *Bacteroidetes*) is interesting. The genus *Bacteroides* seem to play a beneficial role in glucose metabolism where *B. intestinalis* and *B. vulgatus* were decreased in T2D patients, and *B. stercoris* was enriched in patients with diabetes remission (1). The same phenomenon was also noted in experimental animals (1). However, decreased abundance of some *Bacteroides* species, including *B. pleibeius, B. dorei, B. vulgatus,* after using acarbose in newly diagnosed T2D patients was reported to be positively associated with body weight in one study (32). As for rodent studies, colonization of *B. fragilis* was associated with more severe glucose intolerance in HFD-fed mice (24). A recent study, which compared fecal microbiota compositions between T2D patients and non-diabetic individuals, showed that *Bacteroides* was an independent risk factor of the disease by diminishing insulin sensitivity (84).

The effects of metformin on *A. muciniphila* were similar in both human and rodent studies. However, there was an inverse association of *Bacteroides* and metformin use in human and mouse studies in this review. Alterations of many other taxa in humans treated with metformin or acarbose were not the same as in mouse studies, and *vice versa*. The diverse dietary habits, metabolism or inflammatory statuses of host, body sizes and organs in these human and mouse studies might contribute to inconsistent findings of gut microbiota compositions (12, 85). Although the gut microbiota of human

TABLE 11 | Association between specific taxa and mouse metabolic parameters.

Parameters	Association	Specific taxa	Alteration	Drugs	Models	References
Fasting blood glucose	Positive	S_*Bacteroides* spp.	↓	M	HFD/STZ mice	(44)
	Negative	S_*Akkermansia muciniphila*	↑	M	HFD mice	(55)
		S_*Bifidobacterium* spp.	↓	M	HFHSD rats	(57)
Body weight	Positive	G_*Candidatus Arthromitus* G_*Roseburia* G_*Marvinbryantia*	↓	L	HFD/STZ mice	(70)
		S_*Clostridia* sp., S_*Clostridiales* spp. S_*Oscillospiraceae* sp. S_*Erysipelatoclostridium* sp. S_*Anaerotruncus* sp. *G3(2012)* S_*Firmicutes* sp. S_*Bacteroidales* sp.	↓	L	HFD mice	(65)
	Negative	S_*Clostridiales* spp. S_*Oscillospiraceae* sp. S_*Burkholderiales* *bacterium YL45* S_*Akkermansia muciniphila*	↑	L	HFD mice	(65)
		G_*Lactobacillus* G_*Turicibacter* G_*Anaerostipes* G_*Allobaculum* G_*Blautia*	↑	L	HFD/STZ mice	(70)
		G_*Lactobacillus* G_*Turicibacter* G_*Allobaculum*	↑	Sa	HFD/STZ mice	(70)
LDL cholesterol	Positive	G_*Romboutsia*	↓	L	HFD/GK rats	(66)
	Negative	G_*Prevotella*	↑	L	HFD/GK rats	(66)
Total cholesterol	Positive	S_*Prevotella* spp.	↓	M	OLETF rats	(50)
		S_*Clostridia* sp. S_*Clostridiales* spp. S_*Oscillospiraceae* sp. S_*Erysipelatoclostridium* sp. S_*Anaerotruncus* sp. *G3(2012)* S_*Firmicutes* sp. S_*Bacteroidales* sp.	↓	L	HFD mice	(65)
		G_*Romboutsia*	↓	L	HFD/GK rats	(66)
		S_*Clostridium cocleatum*	↑	M	HFD mice	(55)
	Negative	G_*Prevotella*	↑	L	HFD/GK rats	(66)
Triglyceride	Positive	S_*Prevotella* spp.	↓	M	OLETF rats	(50)
		G_*Romboutsia*	↓	L	HFD/GK rats	(66)
	Negative	G_*Prevotella*	↑	L	HFD/GK rats	(66)

G, Genus; S, Species; M, metformin; Sa, saxagliptin; L, liraglutide; ↑, significantly increase; ↓, significantly decrease.

and mice are dominated by two major phyla, i.e., *Bacteroidetes* and *Firmicutes*, approximately 85% of the representative gut microbiota sequences in mice were not found in humans (86).

Further, the genera *Escherichia* and *Shigella*, belonging to the order of *Enterobacterales* in phylum *Proteobacteria*, were found to increase consistently after metformin treatment in T2D patients. Certain bacteria belonging to the phylum *Proteobacteria*, including the order *Enterobacteriales*, was found to be overly present in patients with metabolic disorders and T2D, and were positively related to intestinal permeability and endotoxemia in the pathophysiology of these metabolic diseases (87, 88). Enrichment in the order *Enterobacterales*, especially *Escherichia coli*, was demonstrated to play an important role in gut inflammation in patients with inflammatory bowel disease and also in various mouse model of colitis (88). Elbere et al. (25) observed an association between the severity of gastrointestinal

side effects and increased abundance of the genera *Escherichia* and *Shigella*. Thus, the enrichment of the order *Enterobacteriales* might contribute to gastrointestinal side effects of metformin.

In this review, the results of β-diversity indicate significant changes in gut microbiome structure related to metformin or acarbose treatment. The findings on α-diversity among those treated with metformin were inconsistent while the richness and evenness were decreased after treating with acarbose. For healthy human subjects, the reference microbiome list and abundance profile showed various ratios of *Bacteroidetes* and *Firmicutes*, as well as the other phyla, e.g., *Acinobacteria, Proteobacteria* (89). This might reflect sufficient α- and β-diversities in healthy individuals due to significant regional heterogeneity at the species level and consistency at the higher taxonomic level (89). With regard to T2D patients, the associations between the disease and the diversities of microbiota were inconclusive (1). In the other words, there is no consensus or simple way to make a conclusion on the relationship between diversity and gut microbiota compositions among T2D patients treated with metformin or acarbose.

While metformin and acarbose have been shown to stimulate the growth of SCFA-producing bacteria, e.g., *Lactobacillus* and *Bifidobacterium*, information on the effects of these drugs on the fecal levels of various SCFAs in humans is lacking in the literature. Thus, this review entailed data from mouse studies, which are more informative. These mouse studies showed an increase trend in fecal and cecal levels of acetate, propionate, and butyrate in response to treatments of metformin, acarbose, voglibose, dapagliflozin or canagliflozin, of which the impacts on other physiological functions, other than that in gastrointestinal track, warrant further evaluations (90). With respect to bile acids, information on the effects of anti-hyperglycemic agents on their levels is limited and often conflicting in human vs. rodent studies, which merits further investigations to understand the role of other confounding factors, such as diets, antibiotic therapy, and disease states (91).

Again, the most critical limitation is lack of consistency among human and rodent studies. In humans, differences in the health status of participants, disease type or staging, ethnicity, drug dosage, and duration of treatment might directly impact gut microbiota compositions. Furthermore, it becomes difficult to come to a conclusion due to the small number of participants as well as differences in study design in each study. The risk of bias of studies needs to be taken into considerations, in which overall bias of randomized trials was high risk and unclear risk, and two out of five quasi-experimental studies and all of observational studies were at serious risk. Also, there was a high degree of heterogeneity in rodent studies due to differences in species used and environmental factors, as alluded above. Differences in microbiota analysis methods could also be a cause of deviation.

Another major limitation is lack of human studies on anti-hyperglycemic drugs beyond metformin and acarbose. Thus, no conclusion could be reached regarding the associations between human gut microbiota and these drugs. Because results from rodent models might not always be translatable to humans, conclusions should be made with cautions. Although findings from rodent studies included in this review suggest potential positive effects of other anti-hyperglycemic drugs besides metformin and acarbose on human gut microbiota, additional human studies on these drugs are needed to clarify the role of gut microbiota in their therapeutic efficacies.

In light of the enormous amounts of published data, this systematic review aims to provide readers a comprehensive view of this emerging area by taking an integrated approach through an all-inclusive literature search in conjunction with vigorous data extraction and validation, and assessment of risk bias. Moreover, this systematic review has tried to differentiate various aspects of the anti-hyperglycemic drug-gut microbiome-host axis, thereby filling the gap of merging all available data from human or animal studies relevant to the inter-dependence between anti-hyperglycemic drugs and the specific taxon of gut microbiota. Nevertheless, more investigations are warranted to support the positive contribution of metformin and acarbose to the health of gut microbiome (e.g., *A. muciniphila*, *Lactobacillus*, *Bifidobacterium longum*). In addition, given the limited information available in the literature, more studies are needed to shed light onto the roles of other anti-hyperglycemic drugs (e.g., miglitol, voglibose) in modulating human host taxa of gut microbiome.

CONCLUSION

This review highlights that changes in specific taxa and β-diversity of gut microbiota were associated with metformin and acarbose in humans while pertinent information for other anti-hyperglycemic drugs could be only obtained in rodent studies. These results support the possible action mechanisms of these drugs, which may have translational potential to foster new approaches for the treatment of diseases related to gut dysbiosis in the future. Mouse studies on the other anti-hyperglycemics suggested the links between these drugs and gut microbiota were inconclusive. Therefore, additional human studies are needed to explore the role of gut microbiota in their therapeutic efficacies or side effects.

DATA AVAILABILITY STATEMENT

The original data presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TC, P-CL, H-WL, CC, C-SC, K-CW, J-LH, and L-YY collected, screened, and extracted the data and analyzed the results. TC, H-WL, CC, and L-YY wrote the first draft of the manuscript. All authors contributed to conception, design of the study, manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.573891/full#supplementary-material

Supplementary Figure 1 | Risk of bias of randomized trials. **(A)** Each risk of bias item for each study. **(B)** Each risk of bias item across studies.

Supplementary Figure 2 | Risk of bias of quasi-experimental studies. **(A)** Each risk of bias item for each study. **(B)** Each risk of bias item across studies.

Supplementary Figure 3 | Risk of bias of observational studies. **(A)** Each risk of bias item for each study. **(B)** Each risk of bias item across studies.

Supplementary Figure 4 | Risk of bias of animal studies. **(A)** Each risk of bias item for each study. **(B)** Each risk of bias item across studies.

Supplementary Table 1 | Searching strategy.

Supplementary Table 2 | Characteristics of housing and dietary of studied animals.

Supplementary Table 3 | Effects of anti-hyperglycemia drugs on level of short-chain fatty acids (SCFAs).

Supplementary Table 4 | Effects of anti-hyperglycemia on bile acid levels.

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Gestational Diabetes Is Uniquely Associated With Altered Early Seeding of the Infant Gut Microbiota

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Gestational diabetes mellitus (GDM) is a worldwide public health problem affecting up to 27% of pregnancies with high predictive values for childhood obesity and inflammatory diseases. Compromised seeding of the infant gut microbiota is a risk factor for immunologic and metabolic diseases in the offspring; however, how GDM along with maternal obesity interact to alter colonization remains unknown. We hypothesized that GDM individually and in combination with maternal overweight/obesity would alter gut microbial composition, diversity, and short-chain fatty acid (SCFA) levels in neonates. We investigated 46 full-term neonates born to normal-weight or overweight/obese mothers with and without GDM, accounting for confounders including cesarean delivery, lack of breastfeeding, and exposure to antibiotics. Gut microbiota in 2-week-old neonates born to mothers with GDM exhibited differences in abundance of 26 microbial taxa; 14 of which showed persistent differential abundance after adjusting for pre-pregnancy BMI. Key pioneering gut taxa, including potentially important taxa for establishing neonatal immunity, were reduced. *Lactobacillus*, *Flavonifractor*, *Erysipelotrichaceae*, and unspecified families in *Gammaproteobacteria* were significantly reduced in neonates from mothers with GDM. GDM was associated with an increase in microbes involved in suppressing early immune cell function (*Phascolarctobacterium*). No differences in infant stool SCFA levels by maternal phenotype were noted; however, significant correlations were found between microbial abundances and SCFA levels in neonates. Our results suggest that GDM alone and together with maternal overweight/obesity uniquely influences seeding of specific infant microbiota in patterns that set the stage for future risk of inflammatory and metabolic disease.

Keywords: gestational diabetes, maternal obesity, microbiota, excess gestational weight gain, infant, short-chain fatty acids

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INTRODUCTION

Compelling epidemiological and experimental data show a strong link between maternal metabolic health and the origins of obesity and metabolic disorders in subsequent generations (1). In particular, gestational diabetes mellitus (GDM) and maternal overweight/obesity (OW/OB) independently and together increase offspring risk for metabolic diseases associated with inflammation and weight gain, including obesity, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD) (1, 2). The development of several essential biological systems depends on proper infant gut microbe seeding and expansion; these systems include energy extraction from the diet, gut barrier function, and immune system maturation/education (3, 4). Maternal obesity has been shown to increase neonatal cytokine levels (5) and alter cytokine responsiveness of newborn innate immune cells in early life (6, 7), suggesting that maternal obesity influences childhood risk for metabolic disease, in part through dysregulation of the early immune system. Early colonization of the neonatal gut is a central component to the development of appropriate innate and adaptive immune responses through high levels of *Lactobacillus* (8, 9) and early priming by members of the phylum *Proteobacteria* (10). Multiple studies have shown that maternal obesity alters the composition of the infant gut microbiota throughout the first years of life (11–14); however, only a few data are available in infants born to mothers with GDM.

Recent studies reported that GDM was associated with unique changes in the gut microbiota composition in women during the first trimester of pregnancy (15, 16), third trimester (17–19), and postpartum (17) (as reviewed in (20)). Another study reported that women with GDM had a distinct gut microbiota composition in the second trimester (21), enough to provide discriminatory power to predict GDM status (22). However, no differences in gut microbiota were observed 5-years postpartum in women with previous GDM compared with women without a GDM diagnosis (23). Studies on the microbiota in neonates showed differences in microbe composition between infants born to mothers with and without GDM (24–27). These studies are often confounded by various perinatal conditions known to disrupt offspring microbiota colonization, such as mode of delivery, type of feeding, antibiotic usage, and maternal obesity or excess gestational weight gain (EWG). In addition, maternal diet (28, 29) and host genetics (30, 31) have been reported to impact the infant microbiota, although these are more difficult to determine and quantify in humans. Differences in breast milk composition might also contribute to the infant gut microbiota in breastfed infants (12, 32–34). Variability in the control of confounders in these studies raises caution when interpreting and comparing published data, while illustrating the need for further investigations in order to determine how to best characterize the gut microbiota in infants born to women with GDM.

Despite the explosion of data in the microbiome field, small, well-controlled, hypothesis directed studies have yet to identify the impact of GDM on the infant microbiome. Accordingly, we investigated whether GDM alone and in association with the common comorbidity obesity altered the microbiota colonization pattern and diversity in 2-week-old infants. As a secondary outcome, the influence of EWG alone and in association with OW/OB were also investigated. Short-chain fatty acids (SCFA), mainly acetate, butyrate, and propionate, in the infant gut have potent anti-inflammatory effects, and play an important role in shaping mechanisms underlying immune cell development (35); therefore, infant stool SCFA levels were assessed. In this study, we demonstrate significant decreases in early seeding of pioneer microbes in infants born to mothers with GDM that may play a role in future postnatal immune cell development, and associations between phyla and SCFAs in these infants.

MATERIALS AND METHODS

Study Cohort

Subjects were enrolled in one of three ongoing longitudinal studies of mother-infant dyads between 2012 and 2017 in the Denver, Colorado metropolitan area. All aspects of the clinical studies were approved by the Colorado Multiple Institutional Review Board and the studies were registered at http://www.clinicaltrials.gov (NCT01693406, NCT02244814, and NCT00826904). All research was performed in accordance with relevant guidelines and regulations. Informed consent was obtained from all women and all planned to exclusively breastfeed for at least 4 months and were otherwise healthy. Women with any chronic medical diseases requiring treatment such as cardiopulmonary or renal disease, or pre-existing diabetes were excluded.

Women enrolled in this study were aged 20–39 years old. Maternal pre-pregnancy BMI was based on self-report of pre-pregnancy weight and measured height. Women were classified as normal weight (NW; <25 kg m^{-2}) or overweight/obese (OW/OB; >28 kg m^{-2}) based on pre-pregnancy BMI. GDM was diagnosed by Carpenter and Coustan criteria (36). Briefly, between gestation weeks 24–28, women who failed a non-fasting 1-h blood glucose measurement after 50 g oral glucose solution (≥135 mg dl^{-1}) underwent a 3-h 100 g oral glucose tolerance test. Two or more abnormal blood glucose measurements at fasting, 1, 2, or 3 h per thresholds (≥95, 180, 155, or 140 mg dl^{-1}) were considered positive for GDM. EWG was determined by the change in weight from self-reported pre-pregnancy weight to weight at 36–37 weeks gestation as measured by clinical staff at that study visit. EWG was determined using Institute of Medicine (IOM) standards which have recommended ranges of weight gain according to pre-pregnancy BMI. Women were deemed to have EWG if total weight gain was above IOM recommendations (16 and 9 kg for NW and OW/OB groups, respectively) or no EWG if weight gain was below these thresholds (37). All infants were born of a singleton pregnancy at term (>37 weeks gestation). Our inclusion criteria were vaginal delivery or cesarean delivery after trial of labor (ensuring exposure to the vaginal canal and associated microbiota), no antibiotics or probiotics except in the immediate peripartum period, and predominate breastfeeding in the first 2 weeks of life. Women with GDM were included only if managed by diet alone, without the use of medications (such as insulin or oral agents) to control blood glucose. Thus, we attempted to eliminate the major confounders that typically interfere with early infant microbial colonization of the gut.

Forty-six mother-infant pairs met the inclusion criteria. Infant adiposity (percent fat mass) was measured by air displacement plethysmography in a PEAPOD Infant Body Composition System (COSMED, Rome, Italy) at the 2-week postpartum visit. Of note, four infants were delivered *via* cesarean delivery after a trial of labor, four infants had formula supplement ranging from 2 to 8 ounces per week at 2 weeks of life, one mother had taken probiotics consistently for greater than 2 years, five women were given penicillin at delivery for Group B Streptococcus, and one mother received doxycycline antibiotics during the 2-week postpartum period.

Microbiota Composition

At the 2-week postpartum visit, mothers brought an infant stool sample, collected within 24 h of visit and saved in a frozen diaper at home (~−20°C), which was then aliquoted and stored at −80°C. Profiling of stool bacteria was conducted by 16S rRNA gene sequencing, as outlined previously (12, 38). In brief, microbial DNA from all stool samples was extracted using the PowerFecal DNA Isolation kit (Qiagen, Germantown, MD, USA) per manufacturer's instructions. Isolated DNA was quantified by quantitative PCR for targeted bacterial subgroups as previously (39, 40). Broad-range bacterial 16S rRNA gene amplicons were generated using barcoded primers 27F-YM (5′ - AGAGTT TGATYMTGGCTCAG) and 338R (5′ - TGCTGCCTCCCGTA GGAGT) targeting approximately 300 base pairs of the V1-V2 variable region (39, 41, 42). Primer 27F-YM was designed to enhance amplification of *Bifidobacterium* (41). Pooled amplicons were diluted to 15 pM, spiked with 25% of the Illumina PhiX control DNA, and paired-end sequenced on the Miseq platform with a 600-cycle v.3 reagent kit (Illumina, San Diego, CA, USA). Assembled sequences were aligned and classified with SINA (1.3.0-r23838) (43) using the 418,497 bacterial sequences in Silva 115NR99 (44) as reference configured to yield the Silva taxonomy. Closed-reference operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments. OTUs were analyzed at the genus level, the lowest taxonomic level generated by SINA. Stool microbiota profiling was successful in all samples. The median sequencing depth was 191682 with an IQR of 111622. The mean Good's Coverage was 91.45% with a standard deviation of 2.6%.

SCFA Collection and Measurement

Of the total cohort, we had 23 samples available for SCFA quantification (NW = 12 [GDM-NW = 5, No GDM-NW = 7; EWG-NW = 4, No EWG-NW = 8] and OW/OB = 11 [GDM-OW/OB = 6, No GDM-OW/OB = 5; EWG-OW/OB = 5, No EWG-OW/OB = 6]). Infant stool samples were weighed and diluted to equivalency with acidified water (pH 2.5) containing 1 mmol ethyl-butyric acid per liter as an internal standard. Samples were sonicated for 10 min, incubated at room temperature for 10 min, and then centrifuged at 10,000 × *g* for 10 min at room temperature. Supernatant was collected, re-centrifuged, and stored at −80°C until analysis, as previously (12, 45). Samples were analyzed using a 6890 series gas chromatograph with flame ionization detector (Agilent). Samples were injected at a 10:1 split ratio; the inlet was held at 228°C and the transfer line was held at 230°C. Separation of SCFAs was achieved on a 30m TG-WAX-A column (0.25-mm ID, 0.25-mm film thickness; Thermo Scientific) by using a temperature program as previously (45). Acetate, propionate, and butyrate were quantified using standards of commercially purchased compounds and samples were adjusted for extraction efficiency differences by normalizing to the internal standard.

Statistical Analysis

All data manipulation, analyses, and graphics were conducted using R and RStudio (versions 3.5.1 and 1.1.456, respectively). The R package tidyMicro (version 1.48) was used for all analyses (46) and ggplot2 was used for visualizations (47). Maternal and infant characteristics were summarized with frequencies (%) for categorical variables and means (standard deviations) or median (interquartile range [IRQ]) for continuous variables. Differences in these characteristics were assessed by Fisher's exact test for categorical variables and *t*-tests for continuous variables. Relative abundance (RA) was calculated as the number of sequencing reads of each taxon in a sample, standardized by the total number of sequences generated for that sample. Only taxa that were present in at least 5% of infants and had a RA of a least 0.1% in at least one infant were included in the analyses. Sequence counts for taxa that did not meet these requirements were aggregated into an "Other" category. These filtering requirements were applied at the phylum, family, and genus levels. Sequence counts that could not be classified to the taxonomic level of interest were left as unclassified counts of the lowest level possible. For example, during genus-level analyses, sequence reads only classifiable to a family level were analyzed as unclassified (i.e., unclassified *Lactobacillaceae*).

Alpha diversity measures Chao1, Shannon diversity ($H = -\text{sum}(p_i * \log2(p_i))$, where p_i is the RA of taxon i), and Shannon evenness ($\frac{H}{H_{max}}$) were all calculated through 1,000 replicate re-samplings based on the genus-level OTU counts. The rarefaction level for alpha diversity measures was 57,228, the minimum sequencing depth of this cohort. UniFrac distances weighted by individual taxa counts and the Bray-Curtis dissimilarity were also calculated based on genus-level counts. Differences in infant alpha diversities were assessed using linear regression models and a *t*-test on the regression coefficients. Differences in infant UniFrac distances and beta diversities were assessed using a non-parametric permutation-based multivariate analysis of variation (PERMANOVA) test from the *vegan* package with 999 permutations. We tested for significant impacts from the co-occurrences between maternal GDM and OW/OB as well as maternal EWG and OW/OB for each diversity measure by including interaction terms between the two phenotypes in each model.

Differences in the RAs of phylum-, family-, and genus-level taxa were evaluated using generalized linear models (GLM) assuming a negative binomial distribution and log link function; the total number of sequences was used as an offset. Benjamini and Hochberg's false discovery rate (FDR) correction was then applied to adjust for multiple comparisons within each taxon level with significance defined as FDR p value ≤0.05. For each taxon, a GLM model was first fit including an interaction term between maternal BMI and either GDM or EWG (separate models

for GDM and EWG). If the interaction term was significant (FDR $p < 0.05$), it was determined that the effect of GDM (or EWG) on the taxa was modified by maternal BMI and results were reported for these models. If the interaction term was not significant, the interaction term was removed from the model and the independent effect of GDM (or EWG), after adjusting for OW/OB, was modeled and reported. Estimated relationships between taxa RA and maternal phenotype are reported as a rate ratio (RR), which was obtained by exponentiating the regression coefficients from the GLM models, and Wald confidence intervals were calculated for each RR. Stacked bar charts were constructed using estimated taxa RA obtained from the GLMs to visually display the estimated microbiota compositions by phenotypic group. The top 10 highest estimated overall abundance taxa were plotted while the remaining estimated taxa counts were aggregated into an "Other" category for readability.

Differences in butyrate, propionate, acetate, and total SCFAs were assessed using linear regression models and a *t*-test on the regression coefficients. For each SCFA, we tested for associations between the SCFA and the co-occurrence of maternal GDM and OW/OB as well as maternal EWG and OW/OB using interaction terms. Spearman correlations between SCFA measurements and the centered log ratio of genus-level counts were also calculated. A taxon with a correlation $r \geq |0.5|$ was considered to be clinically meaningful. These correlations were visualized using Rocky Mountain plots, a variation on the Manhattan plot; each line represents the strength of correlation for an individual taxon, with positive correlations extending up from the 0-correlation reference line and negative correlations extending down.

The three longitudinal studies in which samples were collected had limited preliminary data and minimal published data to generate sample size power calculations. Therefore, sample sizes were determined using estimates of microbiota variability and published standard deviations of desired measurements in NW women (12). Specifically, these parent studies were each powered to have 80% power to detect ~18% differences between phylum level abundances in the microbiota from infants of NW and OW/OB women with a Type I error of 0.05, requiring 20 infants per a group. Here, we mitigated Type I error rate inflation through an FDR correction for multiple comparisons. Importantly, the infants selected without exception were delivered vaginally or after a trial of labor, were predominantly breastfed, and with one exception, had no exposure to antibiotics outside of the peripartum period. Thus, we eliminated the major confounders that typically interfere with early infant microbial colonization.

RESULTS

Cohort Characteristics

Forty-six women and their neonates met the inclusion criteria both at birth and at 2-weeks postpartum including vaginal delivery or trial of labor, breastfeeding as the main source of feeding, and no antibiotic exposure (except in the immediate peripartum period). We studied infants born to 13 women with GDM and 33 women without GDM (**Table 1**). Of these 46 women, 27 were NW (BMI = 22.5 ± 2.2 kg/m^2) and 19 were OW/OB (BMI = 32.2 ± 3.9 kg/m^2)

TABLE 1 | Maternal and infant characteristics stratified by maternal GDM.

	GDM	No GDM	*p* value
Maternal			
Weight group, *n* (%)			0.331
NW	6 (46.2)	21 (63.6)	
OW/OB	7 (53.8)	12 (36.4)	
Race/ethnicity, *n* (%)			0.089
Asian, non-Hispanic	4 (30.8)	1 (3.0)	
Black, Hispanic	0 (0)	1 (3.0)	
Black, non-Hispanic	1 (7.7)	2 (6.1)	
White, Hispanic	1 (7.7)	2 (6.1)	
White, non-Hispanic	7 (53.8)	27 (81.8)	
Age, y	32.1 (4.7)	31.5 (3.2)	0.614
Pre-pregnancy BMI, kg m^{-2}	27.6 (5.5)	26.1 (5.8)	0.398
Gestational weight gain, kg	8.9 (4.7)	13.9 (6.3)	0.013
Primiparous, *n* (%)	4 (36.4)	16 (50.0)	0.285
Cesarean delivery*, *n* (%)	2 (15.4)	2 (6.1)	0.323
Fasting glucose, mg dl^{-1}	78.0 (7.6)	77.0 (7.1)	0.688
Missing, *n* (%)	0 (0)	7 (21.2)	
Infant			
Gestational age, week	39.4 (0.9)	40.0 (1.0)	0.064
Birthweight, g	3.24 (0.36)	3.35 (0.51)	0.490
Fat mass, %	11.1 (3.3)	10.0 (4.0)	0.378
Missing, *n* (%)	1 (7.7)	2 (6.1)	
Sex, *n* (%)			1.000
Male	6 (46.2)	16 (48.5)	
Female	7 (53.8)	17 (51.5)	
Formula supplementation, *n* (%)	3 (23.1)	1 (3.0)	0.062

Data are expressed as count (%) or mean (SD).
**After trial of labor.*
p values assessed by Fisher's exact test or chi-square test for categorical variables and t-test for continuous variables.

(**Supplementary Table S1**). Fasting glucose levels at 37 weeks were not significantly different between women with and without GDM (78.0 ± 7.6 and 77.0 ± 7.1 mg/dl, respectively; $p = 0.688$; **Table 1**), indicating excellent fasting glycemic control in our cohort of GDM women. Adiposity of infants born to mothers with and without GDM was not different at 2 weeks of life. Infant adiposity was also not different between infants born to NW and OW/OB mothers (**Supplementary Table S1**) or to mothers with and without EWG (**Supplementary Table S2**). Overall, an even distribution of male and female infants was studied. Sample metadata with variables used in the analyses is shown in **Supplementary Table S3**.

GDM Is Associated With Infant Microbiota Composition

The associations between infant microbiota diversity and GDM status and OW/OB were examined to understand if GDM had an impact on the overall microbiota composition. No significant differences were observed in Shannon $\frac{H}{H_{\max}}$ or Shannon H alpha diversities (measures of genera evenness and complexity, respectively) with GDM either individually or together with OW/OB (**Figures 1A, B**, respectively; **Supplementary Table S4**). The association between the Chao1 measure of richness and maternal OW/OB in infants from mothers without GDM trended higher compared with NW mothers without GDM but did not reach statistical significance ($p = 0.059$). Maternal GDM, OW/OB, and the co-occurrence of these did not significantly impact Bray-Curtis dissimilarity or weighted UniFrac distances, both measures of beta diversity (**Supplementary Table S4**).

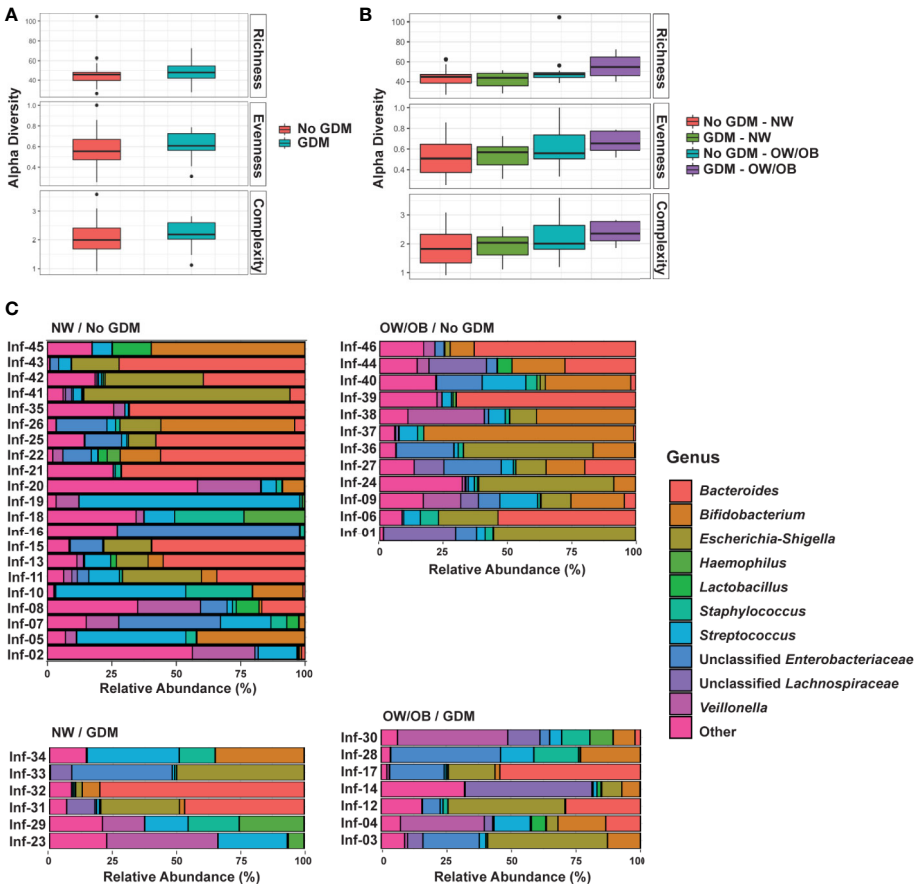

FIGURE 1 | GDM and maternal overweight/obesity alter infant gut microbiota. Box plots of genus-level alpha diversities richness (Chao1), evenness (Shannon $\frac{H}{H_{max}}$), and complexity (Shannon H) by **(A)** GDM and **(B)** GDM and weight group. Black dots represent outliers within strata. **(C)** Relative abundance of microbiota taxa for each infant at the genus level. Genus-level taxa shown are the 10 most abundant with the remaining classified as Other. Relative abundance estimated using negative binomial regression on 16S rRNA gene amplicon sequences from stool samples, shown as percent of total. GDM, gestational diabetes; NW, normal-weight; OW/OB, overweight/obese.

To understand the impact of GDM on individual microbes, we examined associations between the maternal phenotype and RA of individual taxa at the phylum, family, and genus levels. We first explored associations between GDM and RA modified by maternal BMI through interaction terms. If modification was not present (interaction FDR $p < 0.05$), we examined the associations between GDM and RA in additive models excluding the interaction term. After filtering low abundance taxa, we examined 7 phyla, 49 families, and 70 genera. The most abundant genera in the infant microbiota according to GDM status and maternal BMI is shown in **Figure 1C** and abundant phyla and families are shown in **Supplementary Figure 1**. The full data sets for family- and genus-level taxa are shown in **Supplementary Tables S5** and **S6**, respectively. Altogether, we identified 26 microbial taxa across these levels that were differentially abundant in the gut microbiota of infants from mothers with GDM.

No taxa at the phylum level were significantly different between infants born to mothers with and without GDM and modified by OW/OB ($p > 0.05$ for all interaction terms). Going further in the microbial taxonomy, the co-occurrence of GDM and maternal BMI were associated with taxa abundances at the family and genus levels as summarized in **Figure 2** and detailed in **Supplementary Tables S7** and **S8**, respectively. *Lactobacillaceae* ($p < 0.001$), *Erysipelotrichaceae* ($p < 0.001$), and unspecified families in the class *Gammaproteobacteria* ($p = 0.005$) showed decreased abundance with the co-occurrence of GDM and OW/OB, and this co-occurrence was associated with increased Family-XIII-*Incertae-Sedis* ($p = 0.014$) (**Figure 3A**, **Supplementary Table S7**). Associations with significant alterations in microbe abundances were also observed at the genus level for the co-occurrence of GDM and maternal OW/OB (**Figure 3B**, **Supplementary Table S8**). Notably, *Lactobacillus* was decreased ($p < 0.001$) and *Phascolarctobacterium* was increased ($p < 0.001$) in the co-occurrence of these maternal phenotypes. The presence of GDM in NW mothers was associated with RAs of taxa at the family level: decreased *Lactobacillaceae* ($p < 0.001$), *Erysipelotrichaceae* ($p < 0.001$), and unspecified families in *Gammaproteobacteria* ($p < 0.001$) (**Figure 3A**, **Supplementary Table S7**). Associations with

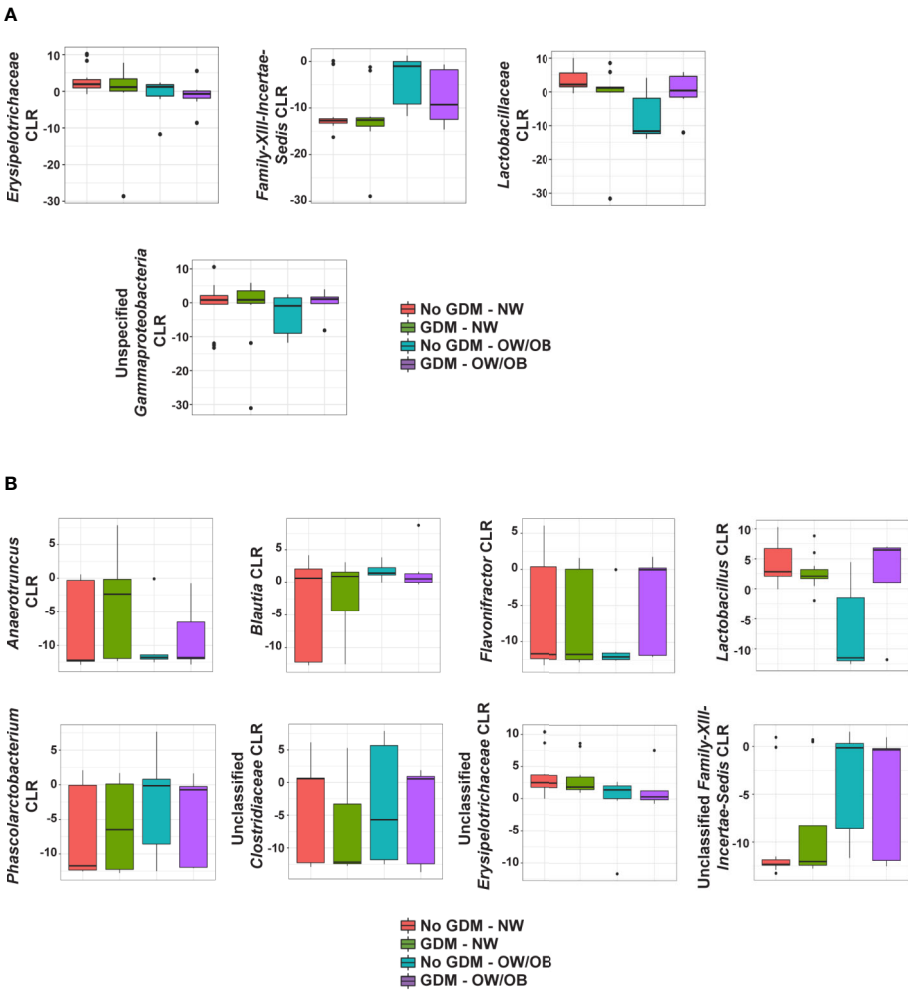

FIGURE 2 | Summary of the impact of maternal GDM on infant gut microbial taxa in the interaction model. Box plots of centered log ratio (CLR) transformed **(A)** family- and **(B)** genus-level taxa counts by maternal GDM status and weight group. Taxa displayed are those whose relationship in infants from mothers with GDM differed significantly based on maternal weight group. GDM, gestational diabetes; NW, normal-weight; OW/OB, overweight/obese.

significant alterations in microbe abundances and GDM were also observed at the genus level including decreased *Lactobacillus* ($p < 0.001$) and *Flavonifractor* ($p = 0.003$) and increased *Phascolarctobacterium* ($p < 0.001$) (**Figure 3B**, **Supplementary Table S8**).

In the additive models, 14 taxa were significantly different in the offspring of mothers with GDM compared to those without, after adjusting for pre-pregnancy BMI (summarized in **Figure 4**). At the phylum level, GDM was associated with decreased *Fusobacteria* ($p = 0.002$) (**Supplementary Table S9**) after controlling for OW/OB. The presence of GDM alone was associated with a number of abundance differences at the family level (**Figure 3A**, **Supplementary Table S10**). GDM after controlling for OW/OB was associated with decreased *Porphyromonadaceae* ($p < 0.001$), *Rikenellaceae* ($p < 0.001$), and *Moraxellaceae* ($p < 0.001$) and an increase in *Lachnospiraceae* ($p = 0.005$). Four genera within *Gammaproteobacteria* were significantly decreased in GDM (**Figure 3B**, **Supplementary Table S11**).

Maternal OW/OB Is Associated With Infant Microbiota Composition After Controlling for GDM

The co-occurrence and additive models also allowed for the investigation of the impact of maternal BMI on infant microbiota in the presence of GDM. The presence of OW/OB in the interaction models comparing the microbiota in infants born to NW mothers without GDM showed a significant decrease in unspecified families in *Gammaproteobacteria* ($p = 0.004$) and an increase in Family-XIII-*Incertae-Sedis* ($p < 0.001$) (**Figure 3A**, **Supplementary Table S7**). Notably, for genus-level taxa, *Phascolarctobacterium* showed increased abundance ($p = 0.005$) (**Figure 3B**, **Supplementary Table S8**). In the additive model, maternal OW/OB alone after controlling for GDM was associated with increased *Fusobacteria* ($p = 0.002$) (**Supplementary Table S9**). Family- and genus-level taxa with associations with maternal OW/OB are shown in **Supplementary Tables S10** and **S11**, respectively.

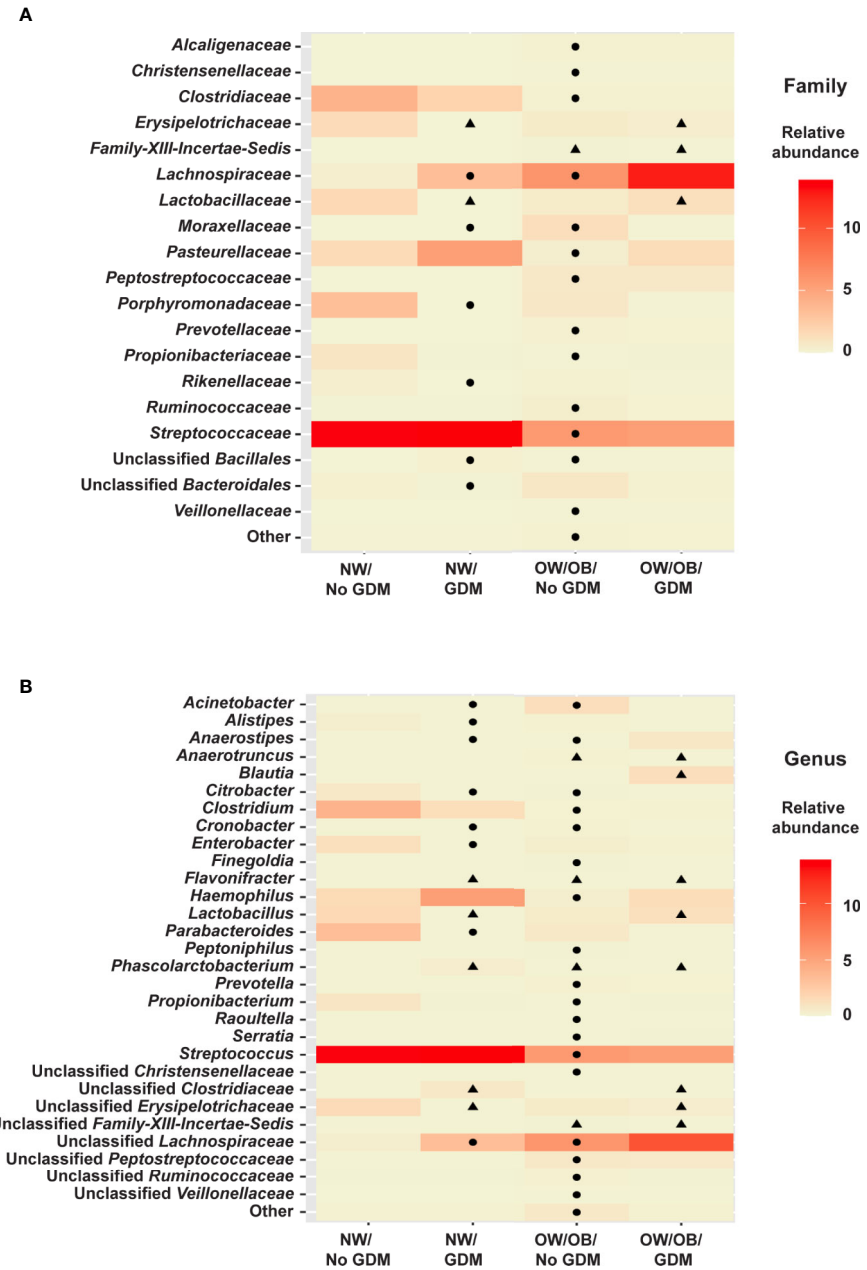

FIGURE 3 | Heat maps of significant relationships between maternal phenotype and infant gut microbiota. Average taxa relative abundances are displayed by maternal phenotype with higher averages colored a deeper red. Cells with a shape inside indicate a significantly different (FDR adjusted p value <0.05) average relative abundance from the NW/No GDM group. Triangles indicate significant differences from the interaction models and circles indicate differences from the additive models for both **(A)** family- and **(B)** genus-level taxa.

Effects of EWG on Infant Microbiota Diversity and Composition

Since EWG beyond recommended limits is common in OW/OB mothers (48) and has been suggested to modify the infant gut microbiota (49, 50), we investigated the effects of maternal EWG, OW/OB, and the interaction of these phenotypes on infant gut microbiota diversity. Increases in Chao1, Shannon $\frac{H}{H_{\max}}$, and Shannon H were all significantly associated with maternal OW/OB after accounting for EWG (p = 0.002, p = 0.031, p = 0.031 respectively; **Supplementary Figure S2A**, **Supplementary Table S12**), but not EWG itself. Similarly, the Bray-Curtis dissimilarity index was altered in infants based on maternal OW/OB (p = 0.043), but EWG neither altered beta diversity nor changed the impact of maternal OW/OB (**Supplementary Table S12**). Maternal EWG,

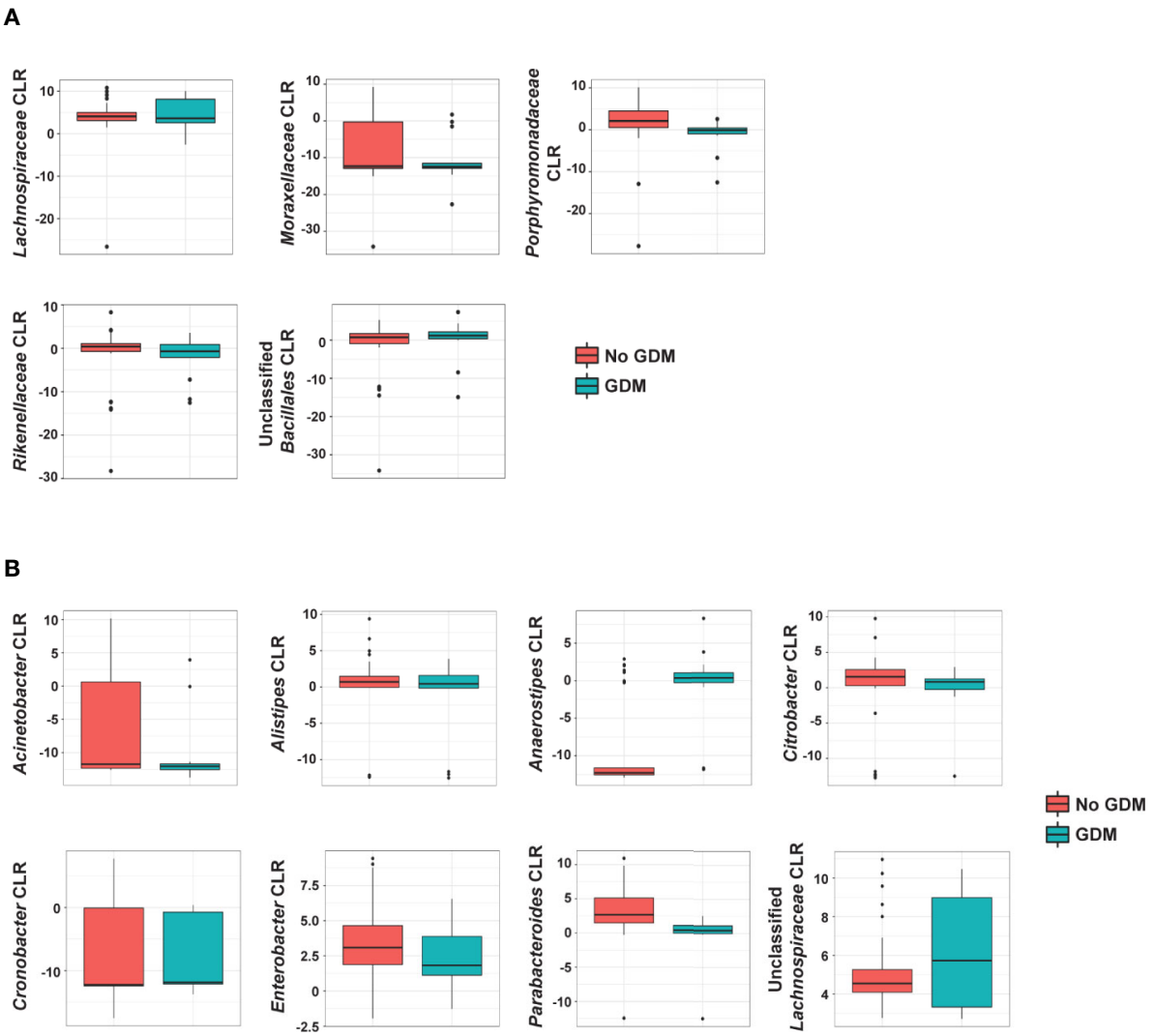

FIGURE 4 | Summary of the impact of maternal GDM on infant gut microbial taxa in the additive model. Box plots of centered log ratio (CLR) transformed **(A)** family- and **(B)** genus-level taxa counts by maternal GDM status. Taxa displayed are those whose abundance changed significantly in infants from mothers with GDM after controlling for maternal weight group. GDM, gestational diabetes.

OW/OB, and the co-occurrence of these did not significantly impact infants' weighted UniFrac distances (**Supplementary Table S12**).

To investigate the impact of EWG on the RA of individual microbes, we followed the same approach as with GDM by first exploring the co-occurrence of EWG and maternal BMI through interactions, and, if not significant, fitting additive models with the two phenotypes. Taxa with the highest estimated RAs of gut microbes in offspring by maternal EWG and OW/OB are displayed at the phylum, family, and genus levels (**Supplementary Figures S2B, S3A, B**, respectively) and the full family- and genus-level data sets are shown in **Supplementary Tables S13** and **S14**, respectively. Overall, we identified 26 taxa that had different RAs of microbes from infants of mothers with EWG (**Supplementary Table S15**). EWG, OW/OB, and the co-occurrence of these conditions analyzed in the interaction model were associated with significant alterations in RAs at the phylum and family levels (**Supplementary Figure S4A** and **Supplementary Tables S16** and **S17**, respectively). Maternal OW/OB and EWG groups showed significant or trending associations with increased abundance of the phylum *Fusobacteria* ($p < 0.001$ and $p = 0.086$, respectively), yet the presence of both OW/OB and EWG was associated with significantly decreased *Fusobacteria* abundance ($p < 0.001$). At the family level, a significantly higher abundance of *Corynebacteriaceae* was associated with the co-occurrence of both EWG and OW/OB ($p < 0.001$) as well as EWG ($p < 0.001$) and OW/OB ($p = 0.025$) alone. Genus-level taxa with significant associations with maternal EWG, OW/OB, and their co-occurrence are shown in **Supplementary Figure S4B** and **Supplementary Table S18**.

Taxa that were not significantly altered by the co-occurrence between EWG and OW/OB were re-analyzed in the additive model.

No significant associations were observed at the phylum level; however, at the family level, both EWG and OW/OB groups were independently associated with RA alterations after controlling for one another (**Supplementary Figure S4A**, **Supplementary Table S19**). Notably, EWG was associated with decreased abundance of *Prevotellaceae* ($p < 0.001$), *Rikenellaceae* ($p < 0.001$), and *Veillonellaceae* ($p = 0.011$) and increased *Porphyromonadaceae* ($p = 0.010$) and *Alcaligenaceae* ($p = 0.006$). Maternal OW/OB was associated with increased *Prevotellaceae* ($p < 0.001$), *Lachnospiraceae* ($p < 0.001$), and *Ruminococcaceae* ($p = 0.006$) and decreased *Propionibacteriaceae* ($p = 0.018$) and *Pasteurellaceae* ($p = 0.019$). Genus-level taxa with significant independent associations with either EWG or OW/OB are shown in **Supplementary Figure S4B** and **Supplementary Table S20**. Of note, maternal OW/OB was associated with an increase in *Finegoldia* ($p = 0.031$) and *Peptoniphilus* ($p < 0.001$) in the phylum *Firmicutes*.

SCFA Levels in Infants Correlate With Gut Microbes

In our cohort, stool collected from infants at 2 weeks of age was dominated by acetate levels followed by propionate and then butyrate at a ratio of approximately 25:2:1 (**Figure 5A**). Maternal phenotypes and their co-occurrences were not associated with alterations in levels of infant stool SCFAs (**Supplementary Tables S21** and **S22**). However, several taxa were correlated with significant changes in SCFA levels. Acetate levels were positively correlated with the RA of *Enterobacter* in the *Gammaproteobacteria* and negatively correlated with *Prevotella* (**Figure 5B**). Propionate was negatively correlated with *Bifidobacterium* (**Figure 5C**). Butyrate did not meaningfully correlate with any taxa (**Figure 5D**). Total SCFAs correlated with the same microbial families as acetate (**Figure 5E**), likely due to the predominance of this SCFA in the infant gut. All SCFA measurements had consistent, though weak, negative correlations with taxa from the phylum *Actinobacteria* and positive correlations with taxa from the phylum *Proteobacteria* (**Supplementary Table S23**).

DISCUSSION

In this study, we examined if GDM alone and together with maternal obesity during pregnancy drive early offspring gut microbial colonization in a carefully selected cohort of full-term, predominantly breastfed, vaginally-exposed infants, without pre- or postnatal antibiotic exposure. We observed differences in abundance of 26 microbial taxa in stool from neonates born to mothers with GDM; 14 of which showed persistent differential abundance after adjusting for pre-pregnancy BMI. Given that obesity is a major driver of insulin resistance, our results suggest that exposures in infants born to mothers with GDM other than insulin resistance contributes to altered offspring microbiota colonization. Whether a unique maternal gut microbiota, breast milk microbiota, or both contributes to infant microbiota colonization, and whether factors in the infant gut make the environment more suitable to disorder/dysbiosis in offspring from mothers with GDM are unknown. Because infants with dysbiotic microbiota are prone to fluctuations in microbial colonization early in life, changes noted at 2 weeks of age may become less dominant over time. In the future, it will also be important to carry out longitudinal studies in these infants to 3 years of life when the microbiota becomes more stable.

A number of key pioneering gut taxa, including *Lactobacillus*, *Flavonifractor*, *Lactobacillaceae*, *Rikenellaceae*, *Erysipelotrichaceae*, and unspecified families in *Gammaproteobacteria*, were decreased in 2-week-old infants from mothers with GDM. Early microbial contact is involved in the initiation and perpetuation of pivotal immune activation and responsiveness that is central to the development of both innate and adaptive immunity (51–53). Various studies have established a correlation between factors that disrupt the gut microbiota during pregnancy and breastfeeding and immune and metabolic disorders later in life (54, 55). For example, microbiota composition shifts were found to occur in infants that may predict the development of type 1 diabetes (56). Vatanen *et al*. demonstrated that *Bacteroides* species in microbiota of infants with high susceptibility to allergies and type 1 diabetes produced a lipopolysaccharide (LPS) subtype that inhibited immunostimulatory activity of *Escherichia coli* LPS *in vitro* (10). Intraperitoneal injection of *E. coli*-derived LPS led to endotoxin tolerance in immune cells and delayed onset of type 1 diabetes in a mouse model, whereas *Bacteroides* LPS was not protective (10). We found reduced *Citrobacter* in offspring from mothers with GDM; this genus is closely related to other taxa in *Enterobacteriaceae*, including *E. coli*, with similar LPS modifications (57). Decreased *Citrobacter* RA suggests a potential for reduced inflammatory or immunomodulatory roles to the host response to endotoxin tolerance or innate training (58). It remains to be seen whether specific genera or a combination of species imparts different effects on the development of immunity in the infant, as there is enormous diversity and interplay within members of these families. In this regard, however, we recently showed that microbiota from 2-week-old infants born to obese mothers increased obesity and NAFLD in gnotobiotic mice, due in part to increased gut permeability and the inability of bone-marrow derived macrophages to engulf bacteria and protect against inflammation (38, 59). This illustrates that alterations in gut microbiota have functional consequences through the programming of fundamental pathways involved in innate immunity and obesity prior to disease.

Simultaneous exposure to GDM and OW/OB was associated with a decrease in *Lactobacillaceae*, potentially contributing negatively to early immunological development as this taxon was previously shown to contribute to innate immune system education in early life (60). This is consistent with data showing that the genus *Lactobacillus* is reduced in meconium from infants born to mothers with GDM (24). In addition, infants exposed to GDM and OW/OB independently and simultaneous GDM and OW/OB exposure had increased *Phascolarctobacterium*. This genus was previously shown to be elevated in women with GDM compared with pregnant women with normal glucose status (18, 22), suggesting it is

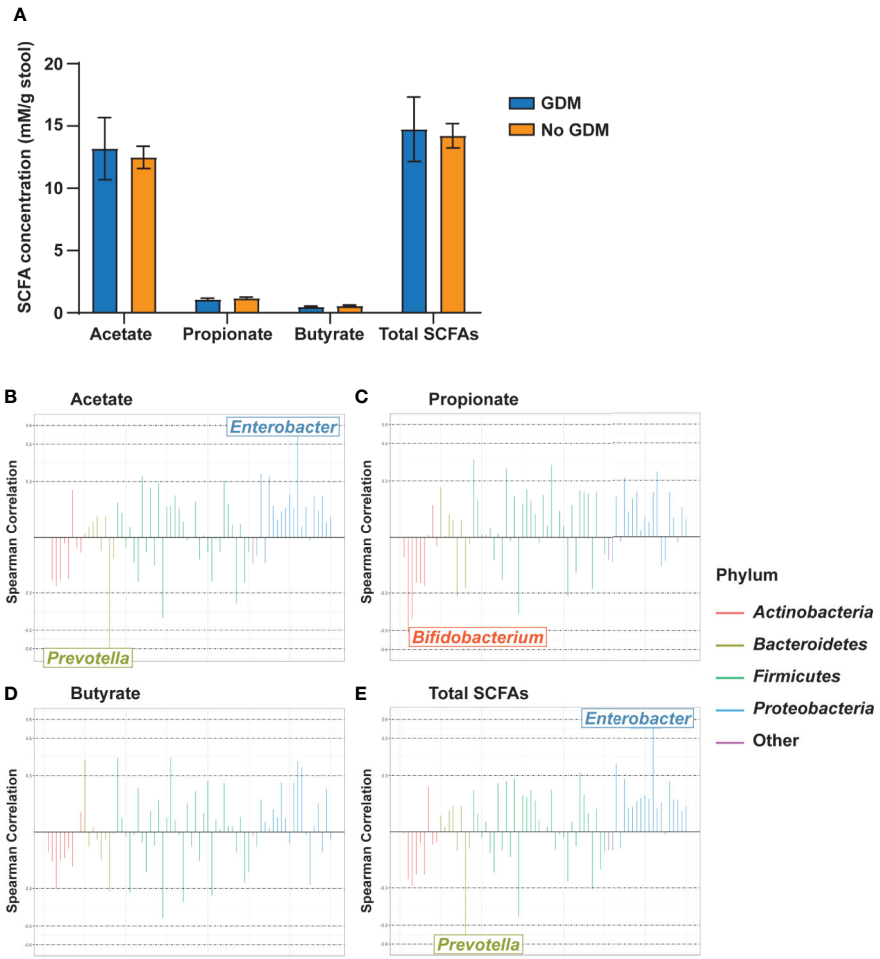

FIGURE 5 | Gut microbiota genus-level taxa correlate with infant stool SCFA levels. **(A)** Infant stool SCFA levels by maternal GDM status. Rocky Mountain Plots representing **(B)** acetate, **(C)** propionate, **(D)** butyrate, and **(E)** total SCFA measurements with a center log ratio transformation of genus-level counts. Correlations were derived using Spearman Correlation with $r > |0.5|$ indicating a meaningful correlation. $n = 23$ for each measure.

vertically transmitted from mother to infant. Interestingly, *Phascolarctobacterium* has been shown to influence intestinal inflammation through its role as a succinate consumer (61). Infants born to mothers with GDM showed a significant association with elevated *Lachnospiraceae* after controlling for OW/OB. *Lachnospiraceae* is enriched in women with GDM (15, 17, 18) and in adults with type 2 diabetes (62, 63), suggesting that it is a marker associated with impaired glycemic control transmitted to infants of GDM mothers.

Additive models of taxa RA demonstrated a prominent role for OW/OB in independently shaping offspring microbiota. OW/OB alone was associated with shifts in microbes, previously reported in infants of OW/OB mothers (11), including decreased *Pasteurellaceae* and increased *Finegoldia* and *Peptoniphilus* after controlling for either GDM or EWG. The increased *Lachnospiraceae* and *Ruminococcaceae* associated with OW/OB (after controlling for either GDM or EWG) have previously been positively correlated with leptin levels (both families), maternal BMI (*Lachnospiraceae*), and insulin levels (*Ruminococcaceae*) in pregnant obese women (64), implying maternal transfer to the infant during delivery and/or lactation. After controlling for either GDM or EWG, infants of OW/OB mothers also have decreased *Streptococcaceae*, which is consistent with our previous study of 2-week-old infants of obese mothers (12). Analysis of EWG (after controlling for OW/OB) found an association with increased *Porphyromonadaceae*; a family previously associated with hepatic steatosis and inflammation in inflammasome-deficient mice (65) as well as in human NASH (66).

No differences in SCFAs by maternal phenotype were noted in our study. However, we did find significant correlations between microbial RAs and SCFA levels among predominantly/exclusively breastfed neonates. For example, acetate levels were positively correlated with the RA of *Enterobacter* in *Gammaproteobacteria*. The formation of acetate by *E. coli*, a pioneering aerobe in *Gammaproteobacteria*, is a commonly observed phenomenon (67, 68); however, RA of *Gammaproteobacteria* was reduced in

OW/OB and GDM infants, suggesting multiple sources for acetate. Acetate can also be consumed by many rapidly proliferating gut microbes, possibly leading to changes in microbial community composition, which in turn could influence host metabolism.

In our study, we used the V1-V2 region for sequencing the 16S rRNA genome. It is important to recognize that these short reads (using one to three variable regions) are unable to accurately and confidently discriminate species-level taxonomic classification (69) and may have underestimated the relative levels of *Bifidobacterium* in the infants (70). We acknowledge that analysis of microbial communities at the species level could provide a very different perspective to the one afforded by genus-level abundance estimates. Our results are limited to offspring from mothers whose GDM was controlled by diet. The role of insulin and metformin on the maternal gut microbiome and SCFA levels in mothers and infants remains to be studied. We did not measure the breast milk microbiome, and this deserves further study.

Overall, many of our findings in the microbiota of infants from mothers with GDM mirror those in maternal GDM gut dysbiosis (17, 21) and those found in infants with compromised immunological function (9, 71). Regarding possible mechanisms for infant prenatal colonization in mothers with GDM, speculation suggests that the intrauterine maternal microbiota is a source of first colonizers in the infant gut, but the existence of an *in utero* microbiota remains controversial. A recent study suggests that microbial products or metabolites are detected in the human fetal intestine and drive *in utero* immune development and education (72); however, the source of the bacteria is unknown. Gestation-only colonization of mice with *E. coli* was reported to modify the intestinal mucosal innate immune system and transcriptome of the offspring (73). Similarly, in a prospective cohort of 26 mother-infant dyads, a high-fat maternal gestational diet was associated with distinct variations in the neonatal gut microbial composition (meconium), which persisted to 4 to 6 weeks of age (74), suggesting a maternal diet-driven microbiota during development. Given differences in early colonizers in 2-week-old offspring of mothers with GDM, it is not unlikely that the neonatal microbiome and immune system may be programmed by maternal factors at different stages of development depending on the cell type and location (75). This may include changes in the maternal microbiome, human milk oligosaccharides, SCFAs or inflammation in mothers with GDM (17, 19, 76).

In conclusion, these results demonstrate an independent role of maternal GDM in the infant gut microbial composition. Additional research in large, prospective, human birth cohorts (both maternal and infant) will be necessary to understand how these early microbiota changes may interact with host components to drive aspects of infant immune development and disease pathways over time in offspring from mothers with GDM. Small changes may become less dominant over time; however, early patterns of microbial succession over the first year of life have been correlated with susceptibility to immune diseases later in life (51–53). This represents a significant challenge but a unique opportunity to discover pathways to developmental programming in the neonate.

DATA AVAILABILITY STATEMENT

16S rRNA gene sequences and associate metadata generated and analyzed during the current study have been deposited in the NCBI Sequence Read Archive and are available through BioProject ID: PRJNA558340. https://www.ncbi.nlm.nih.gov/sra.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Colorado Multiple Institutional Review Board, University of Colorado Anschutz Medical Campus, Aurora, CO, United States. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TS, RJ, and JF wrote the manuscript. TS, TW, and DI performed the experiments. CC, CR, and MK performed bioinformatic and biostatistical analysis of the data. RJ formatted the manuscript, tables, and figures. BY, NK, TH, and LB designed and conducted the clinical studies from which samples were generated. JF and DF provided guidance on the study design, data interpretation, and analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.603021/full#supplementary-material

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ORIGINAL RESEARCH
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Six-Week Exercise Training With Dietary Restriction Improves Central Hemodynamics Associated With Altered Gut Microbiota in Adolescents With Obesity

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Purpose: Obesity in children and in adolescents can lead to adult cardiovascular diseases, and the gut microbiota plays a crucial role in obesity pathophysiology. Exercise and diet interventions are typical approaches to improve physical condition and to alter the gut microbiota in individuals with obesity. However, whether central hemodynamic parameters including subendocardial viability ratio, the augmentation index standardized to a heart rate of 75/min (AIx75), resting heart rate, and blood pressure, correlate with gut microbiota changes associated with exercise and diet is unclear.

Methods: Adolescents (n = 24, 12.88 ± 0.41 years) with obesity completed our 6-week program of endurance and strength exercises along with dietary restriction. Blood and fecal samples were collected, and physical parameters were measured before and 24 h after the last session of the intervention program. Pulse wave analysis using applanation tonometry provided the subendocardial viability ratio, a surrogate measure of microvascular myocardial perfusion, and AIx75, a measure of arterial stiffness and peripheral arteriolar resistance. Correlation analysis detected any associations of anthropometric or central hemodynamic parameters with gut microbiome composition.

Results: Exercise and diet interventions significantly reduced body weight, body mass index, body fat, and waist-to-hip ratio, and lowered levels of fasting blood glucose, serum triglycerides, and high-density lipoprotein cholesterol. AIx75 and resting heart rate were also significantly reduced after the intervention without changes to systolic or diastolic blood pressure. The ratio of intestinal microbiota *Firmicutes* to *Bacteroidetes* displayed a marked increase after intervention. Interventional changes in gut microbiota members were significantly associated with anthropometric and metabolic parameters. Microbial changes were also significantly correlated with central hemodynamic parameters, including subendocardial viability ratio, AIx75, and resting heart rate.

Conclusion: Exercise and diet interventions significantly improved measures of central hemodynamics, including subendocardial viability ratio, AIx75, and resting heart rate, which were correlated with altered gut microbiota in adolescents with obesity. Our findings shed light on the effects and mechanisms underlying exercise and diet interventions on obesity and suggest this approach for treating patients with both cardiovascular disease and obesity.

Keywords: exercise, dietary restriction, obese adolescents, gut microflora, subendocardial viability ratio

INTRODUCTION

Obesity-related cardiovascular disease in children and adolescents is becoming more prevalent and is being accompanied by a rapid increase in childhood and adolescent obesity in wealthy societies (1). It has been shown that childhood and adolescent body mass index (BMI) is positively correlated with the risk of cardiovascular disease in adulthood, indicating that cardiovascular damage may begin during childhood (2). The intestinal microbiota has been recently suggested as an environmental factor involved in the control of body weight and energy homeostasis (3). Accumulating evidence has demonstrated that the gut microbiota plays a crucial role in obesity pathophysiology (4). The intestinal flora is dominated by two microbial phyla, *Firmicutes* and *Bacteroidetes* (5). Previous studies have shown that a higher *Firmicutes*-to-*Bacteroidetes* (F/B) ratio correlates with obesity (6).

Clinical trials have reported that a combined exercise and diet intervention improves the obesity-associated cardiovascular biomarkers of body composition, blood pressure, dyslipidemia, insulin resistance, and inflammation (7, 8). Additionally, Woo et al. have demonstrated that exercise or dietary modification may improve arterial and cardiac function (9). More recently, studies have revealed that physical exercise or diet influences the gut microbiota in individuals with obesity (10–13). In addition, Menni et al. reported that gut microbiome diversity is inversely associated with arterial stiffness in middle-aged women (14). However, it has not yet been investigated whether central hemodynamic parameters correlate with gut microbiota under the lifestyle modifications of exercise and diet.

Thus, in the present study, the effects of a 6-week program of combined endurance and strength training exercise and dietary restriction on central hemodynamic parameters and gut microbiome composition was assessed in adolescents with obesity. Furthermore, the relationship between changes in central hemodynamics and gut microbiota was determined. We hypothesized that the exercise and diet intervention would improve central hemodynamics associated with altered gut microbiota.

MATERIALS AND METHODS

Participants and Ethical Approval

Between July and August 2018, participants with obesity who were 9 to 16 years of age were recruited from a weight loss camp after a physical examination. For inclusion, participants satisfied the obesity diagnosis criteria based on the 2004 report titled "Body mass index reference norm for screening overweight and obesity in Chinese children and adolescents" (15) with a cutoff point for obesity at 95% of the national BMI reference norm. Participants with metabolic, gastrointestinal, or cardiac diseases were excluded. None of participants were using medications known to affect energy expenditure or were losing weight in the previous 3 months whether by dieting, taking weight-loss medication, or engaging in more physical exercise than usual.

Exercise and Diet Intervention

The full details of the exercise and diet intervention have been published in our previous studies (16, 17). In brief, participants were provided with a calorie-restricted but nutritionally complete diet based on their age. The diet consisted of balanced proportions of 60% carbohydrate, 20% protein, and 20% fat, and calories were distributed as 30%, 40%, and 30% at breakfast, lunch, and dinner, respectively. Dietitians prepared and supervised all meals. Participants performed an intense exercise program of 5 h/day, 6 days/week for 6 weeks, which was conducted from 8:00 to 9:30 AM, from 10:00 to 11:30 AM, and from 3:00 to 5:00 PM daily (18, 19). The exercise primarily consisted of endurance training (including bicycling, walking, running, dancing, and ball games) and strength training. The endurance exercises involved moderate- and high-intensity training. Moderate-intensity exercise was defined as 70% to 85% of the participant's maximum heart rate (HRmax), which was calculated using the following formula: 208 − (0.7 × age). High-intensity exercise (approximately 90% of HRmax) was alternated with low-intensity exercise (approximately 60% of HRmax) during training. Strength exercise was performed at 40% to 50% of each participant's maximal strength for two to three sets of 12 to 15 repetitions each, with 2 to 3 min rest between each set. A daily training log assessed the participants' compliance. Measurements were performed before exercise training and 24 h after the last session of the 6-week exercise and diet program.

Body Composition and Biochemical Factors

Height and weight were determined, and BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Body composition was assessed by a body composition analyzer (Inbody 370, Biospace, Seoul, Korea). Blood samples were collected for the assessment of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting glucose levels.

Resting Heart Rate and Brachial Blood Pressure

Resting heart rate (HR), brachial systolic blood pressure (SBP), and diastolic blood pressure (DBP) were obtained using a sphygmomanometer (Omron, 705 IT, Omron Health Care, Japan). Measurements were made in triplicate, and the average value was used.

Pulse Wave Analysis

Pulse wave analysis was noninvasively performed using applanation tonometry (SphygmoCor, AtCor Medical, Sydney, Australia) to obtain the subendocardial viability ratio (SEVR) and wave reflection data. SEVR is a surrogate measure of microvascular myocardial perfusion and was calculated as the ratio of the pressure area under the curve during diastole to the pressure area under the curve during systole. Low SEVR levels indicate reduced subendocardial perfusion. Augmentation pressure is expressed as the difference between the second and first systolic peaks of the ascending aortic waveform. The augmentation index (AIx) was assessed with the SphygmoCor device as a percentage of the ratio of augmentation pressure to aortic pulse pressure. To exclude to influence of heart rate on the AIx, AIx is normalized to a standard heart rate of 75 beats/min (AIx75). AIx75 is considered a measure of arterial stiffness and peripheral arteriolar resistance.

Fecal Sample Collection and 16S rDNA Sequencing

To assess the intestinal flora of each participant, we collected the intestinal contents of each individual before exercise (3 days after enrollment) and after intervention (1 day after 6 weeks of the diet and exercise intervention) (20). After each defecation, the contents of the intestinal tract were placed in separate sterile fecal collection tubes (5 mL), and liquid nitrogen was immediately poured into the tubes for preservation.

Fecal samples were used to obtain total genomic DNA samples for sequencing of the 16S rRNA gene. A library for sequencing was prepared according to Illumina's 16S Metagenomic Sequencing Library Preparation Guide. The library quality was determined using a Qubit 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. The Illumina MiSeq platform sequenced the hypervariable regions V3–V4 of the 16S rRNA gene and generated the raw data.

Cutadapt (version 2.8) was used to remove low-quality readings and adapter sequences from the raw data (**Figure S1**). The software FLASH (version 1.2.11) (21) is used for sequence splicing, and the paired reads obtained by pair-end sequencing are assembled into a sequence by using the overlap relationship to obtain the tags of the hypervariable region. Use the software VSEARCH (version 2.3.4) (22) to cluster the spliced Tags into operational taxonomic units (OTUs). After obtaining the OTU representative sequence, the OTU representative sequence is compared with the database for species annotation through RDP Classifier (version 2.2) software (23), and the confidence threshold is set to 0.8. The comparison database includes: Silva (version 128) (24), Greengenes (version 201305) (25), and UNITE (version 6 20140910) (26). After the spliced tags are optimized, they are clustered into OTUs for species classification with 97% similarity, and the abundance information of each sample in each OTU is counted. The Venn diagram can be used to show the number of common and unique OTUs of multiple samples, and visually show the overlap of OTUs between samples. Combined with the species represented by OTU, the core microorganisms in different environments can be found. QIIME 2 (version 2019.4) (27) calculates classification summary and alpha and beta diversity indicators, statistical analysis, and classification. R script was used for downstream analysis, including rank abundance curves (most abundant ranked 1, next most abundant ranked 2, etc.) based on operational taxonomic units (OTUs), principal component analysis (using ade4 package), a Venn diagram (using VennDiagram package), and alpha (using mothur package) and beta diversity analyses of the OTU abundance.

Statistical Analysis

Linear discriminant analysis (LDA) effect size was used to estimate the magnitude of the impact on each taxonomic group represented by differences pre- and post-intervention. These analyses were represented by bar graphs, the parameters were set to the default P-value < 0.05, and the LDA score of LEfSe was 2.0 (28). Analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Paired-sample t-tests were used to compare the data obtained before and after the intervention. Pearson correlation analysis was performed to measure the association between two linear quantitative measures. Data are shown as mean ± SE. A P-value < 0.05 is used to indicate statistical significance.

RESULTS

Effects of Exercise and Diet Intervention on Anthropometric and Metabolic Parameters

Twenty-four participants (15 males, 9 females) met the criteria for inclusion, completed the program, and provided valid measurements at both baseline and post-training.

Exercise and diet interventions may affect many parameters in our body, for example, anthropometric and metabolic parameters. As shown in **Table 1**, in response to the exercise and diet intervention, participants who had been classified with obesity had significantly reduced body weight (before: 82.64 ± 3.40 kg vs. after: 74.00 ± 3.56 kg, $P < 0.001$), BMI (before: 31.02 ± 0.94 kg/m^2 vs. after: 27.78 ± 0.88 kg/m^2, $P < 0.001$), body fat (before: 34.35 ± 2.05 kg vs. after: 26.30 ± 1.73 kg, $P < 0.001$), and waist-to-hip ratio (before: 0.93 ± 0.01% vs. after: 0.88 ± 0.12%, $P < 0.001$). Participants with obesity also showed significantly lower levels of fasting blood glucose (before: 4.37 ± 0.08 mmol/L vs. after: 4.14 ± 0.07 mmol/L, $P < 0.05$), serum TGs (before: 4.15 ± 0.18 mmol/L vs. after: 3.25 ± 0.07 mmol/L, $P < 0.001$), and LDL-C (before: 2.30 ± 1.14 mmol/L vs. after: 1.73 ± 0.08 mmol/L, $P < 0.001$) following the intervention. However, no significant

TABLE 1 | Anthropometric, metabolic, and hemodynamic parameters of participants before and after 6-week combined exercise and diet intervention.

Parameter	Before (mean ± SE)	After (mean ± SE)	*P*-value
Age (year)	12.88 ± 0.41	–	–
Gender (n) (M/F)	24 (15/9)	–	–
Body weight (kg)	82.64 ± 3.40	74.00 ± 3.56	<0.001
BMI (kg/m^2)	31.02 ± 0.94	27.78 ± 0.88	<0.001
Body fat (kg)	34.35 ± 2.05	26.30 ± 1.73	<0.001
Waist-to-hip ratio (%)	0.93 ± 0.01	0.88 ± 0.12	<0.001
Blood glucose (mmol/L)	4.37 ± 0.08	4.14 ± 0.07	0.014
Triglycerides (mmol/L)	4.15 ± 0.18	3.25 ± 0.07	<0.001
Cholesterol (mmol/L)	0.70 ± 0.11	0.76 ± 0.06	0.576
HDL-C (mmol/L)	1.15 ± 0.05	1.16 ± 0.05	0.790
LDL-C (mmol/L)	2.30 ± 1.14	1.73 ± 0.08	<0.001
SEVR (%)	112.21 ± 6.89	163.08 ± 8.61	<0.001
AIx75 (%)	9.45 ± 1.49	3.13 ± 2.18	0.006
Resting HR (beats/min)	77.08 ± 2.87	60.88 ± 2.65	<0.001
SBP (mm Hg)	114.54 ± 2.51	110.79 ± 1.85	0.079
DBP (mm Hg)	66.75 ± 1.82	63.79 ± 1.99	0.104

BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SEVR, subendocardial viability ratio; AIx75, augmentation index standardized to a heart rate of 75/min; HR, resting heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure.

changes in levels of serum TC (before: 0.70 ± 0.11 mmol/L vs. after: 0.76 ± 0.06 mmol/L, $P = 0.576$) or HDL-C (before: 1.15 ± 0.05 mmol/L vs. after: 1.16 ± 0.05 mmol/L, $P = 0.790$) were found (**Table 1**).

Effects of the Exercise and Diet Intervention on Central Hemodynamics

To investigate the effects of the exercise and diet intervention on central hemodynamics, we next performed pulse wave analysis. Compared with the SEVR in normal conditions (130–200%) (29, 30), individuals with obesity had lower SEVR values (112.21 ± 6.89%). After the intervention, these individuals displayed significant augmentation in their SEVR (163.08 ± 8.61%, $P < 0.001$). A previous study reported the normal and reference value of AIx75 was 9.4 ± 7.7% for age < 34 years (31). After the intervention, these individuals displayed significant reductions in AIx75 (before: 9.45 ± 1.49% vs. after: 3.13 ± 2.18%, $P = 0.006$) and resting heart rate (before: 77.08 ± 2.87 beats/min vs. after: 60.88 ± 2.65 beats/min, $P < 0.001$) (**Table 1**). However, there were no significant changes in levels of SBP (before: 114.54 ± 2.51 mmHg vs. after: 110.79 ± 1.85 mmHg, $P = 0.079$) and DBP (before: 66.75 ± 1.82 mmHg vs. after: 63.79 ± 1.99 mmHg, $P = 0.104$) following the exercise and diet intervention (**Table 1**).

Effects of the Exercise and Diet Intervention on Intestinal Microbiota Composition

To evaluate gut microbial composition, high-throughput DNA sequencing using V3-V4 16S rDNA libraries was performed with fecal DNA from participants before and after the diet and exercise intervention. We obtained a total of 1,574,058 tags, with an average of 32,792 tags per sample. There were 956 OTUs in a total of 48 samples. Pre-intervention, there were 901 OTUs, and post-intervention there were 934 OTUs. We detected 22 unique OTUs pre-intervention and 55 unique OTUs post-intervention (**Figure 1A**). The OTU rank abundance curve (**Figure 1B**) shows species diversity in a sample that simultaneously explains the richness and uniformity of the species contained in the sample. We found that each sample had good species richness and uniformity. The species accumulation boxplot of the 48 samples is shown in **Figure 1C**. As the sample size increased, no significant increase was detected in the microbial species present, indicating that the sample size used in the present study was sufficient to capture species diversity. Rarefaction, a statistical technique, was used with measures that estimate species evenness and richness across samples, the Simpson index (range, 0–1, with higher values indicating less diversity) and the Shannon index (value increasing with the number of overall species and as the distribution of species across samples becomes even). The results showed that both indices increased with sequencing and flattened as the number of sequences sampled increased (**Figure S2**), suggesting that the sequencing data were sufficient to cover nearly all microbiota species in all samples and to reveal accurate microbiological information. In addition, beta diversity was calculated, and the results were plotted in Nonmetric Multidimensional Scaling (NMDS) 3D (**Figure S3**). However, our results showed that no clustering was observed using the ANOSIM test ($P = 0.721$).

We next evaluated the composition of the gut microbiota at the phylum level. The most abundant phyla were *Bacteroidetes* (53.24%) and *Firmicutes* (40.50%), followed by *Fusobacteria* (2.92%), *Proteobacteria* (2.61%), *Actinobacteria* (0.26%), *Tenericutes* (0.25%), *Verrucomicrobia* (0.08%), *Lentisphaerae* (0.04%), *Cyanobacteria* (0.04%), *Synergistetes* (0.01%), and *Saccharibacteria* (0.004%) (**Figure 2**). *Firmicutes* and *Bacteroidetes*, two major phyla of the domain *Bacteria*, are dominant in gut microbiota. Thus, the F/B ratio has been extensively examined for human gut microbiota, including to assess the flora of individuals with obesity. Numerous studies have shown that the F/B ratio is correlated with obesity and with other diseases. Therefore, we analyzed the dominant strains at the phylum level pre- and post-intervention. We found that the level of *Firmicutes* decreased whereas the level of *Bacteroidetes* increased after the diet and exercise intervention. The F/B ratio was significantly decreased after the intervention (post-intervention vs. pre-intervention: 0.737 ± 0.09 vs. 1.11 ± 0.205; n = 24 in each group; $P = 0.011$).

We further compared the alpha diversity pre- and post-intervention to assess changes in intestinal flora diversity and abundance after the intervention (**Table 2**). The average number of observed species (Sobs) was significantly increased (before: 203.59 ± 10.53 vs. after: 240 ± 12.00, $P = 0.002$), indicating that the abundance of intestinal flora post-intervention was superior to that pre-intervention. The Chao and Ace diversity indexes indicate the species richness of the samples, with higher values indicating greater species richness. The Shannon and Simpson indexes indicate the species uniformity and richness of the samples. An increased Shannon index or a decreased Simpson index represents higher population diversity. Of these estimators,

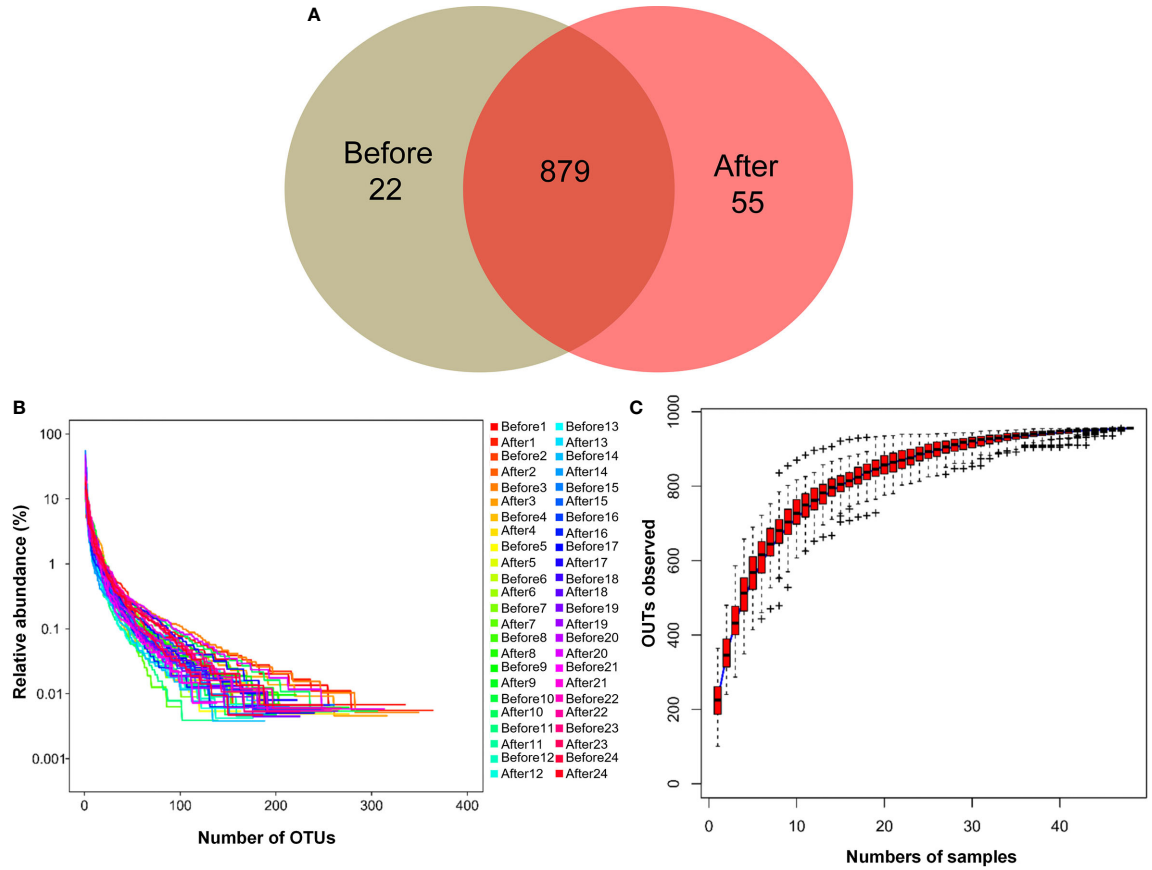

FIGURE 1 | Characteristics of two microbiota. **(A)** Venn diagram. The Venn diagram shows the unique operational taxonomic units (OTUs) pre-intervention (Before; brown) and post-intervention (After; light-red), and the number of shared OTUs (dark-red) pre- and post-intervention. **(B)** OTU rank abundance curves. The legend indicates the Sample Number. Before presents pre-intervention. After presents post-intervention. **(C)** Species accumulation boxplot of the 48 samples.

we found that the Chao (before: 259.77 ± 14.15 vs. after: 299.77 ± 16.33, $P = 0.007$), Ace (before: 265.66 ± 15.33 vs. after: 298.70 ± 14.87, $P = 0.023$), and Shannon indexes (before: 2.93 ± 0.09 vs. after: 3.19 ± 0.10, $P = 0.009$) were significantly increased, whereas the Simpson index (before: 0.14 ± 0.02 vs. after: 0.11 ± 0.01, P = 0.035) was significantly decreased after the intervention.

We further explored the differentially rich taxonomic groups by using LDA effect size as a biomarker discovery algorithm. In addition to uncultured bacterium, we found 12 classified biomarkers, of which 5 were rich pre-intervention, and 7 were rich post-intervention (**Figure 3A**). The LDA scores indicated that the relative abundances of *Lactobacillales, Bacilli*, *Streptococcaceae*, *Streptococcus*, and *Veillonella* were higher pre-intervention than post-intervention (**Figures 3A, B**). These five microorganisms are all members of *Firmicutes* phylum. By contrast, the results showed higher relative abundances of *Lentisphaeria*, *Victivallales*, *Victivallaceae*, *Victivallis*, *Christensenellaceae*, *Christensenellaceae* R-7 group, and *Butyricimonas* post-intervention vs. pre-intervention (**Figures 3A, B**). These seven microorganisms all belong to the *Bacteria* domain. These results indicate that the exercise and diet intervention had a marked effect on bacteria.

Significant Associations Between Changes in Gut Microbiota Members and Metabolic Parameters After the Exercise and Diet Intervention

As shown in **Figure 4A** and **Table S1**, changes in TGs, serum TC, and LDL-C were positively associated with change in the relative abundance of *Family XIII* UCG-001. The change in TC was also positively associated with the changes in *Alloprevotella*, *Eubacterium coprostanoligenes* group, and *Ruminococcus torques* group. By contrast, there was a negative association between the change in TC and the change in *Bacteroides*. A negative association was also found between the change in HDL-C and the change in *Ruminococcaceae* UCG-003. The change in LDL-C was positively associated with the change in *Odoribacter*. The change in fasting glucose level was positively correlated with changes in the *Eubacterium ruminantium* group and *Paraprevotella*, but negatively correlated with change in the *Eubacterium ventriosum* group. Taken together, these results suggested there is a significant correlation between improved lipid profiles and changed gut microbiota composition following the present exercise and diet intervention.

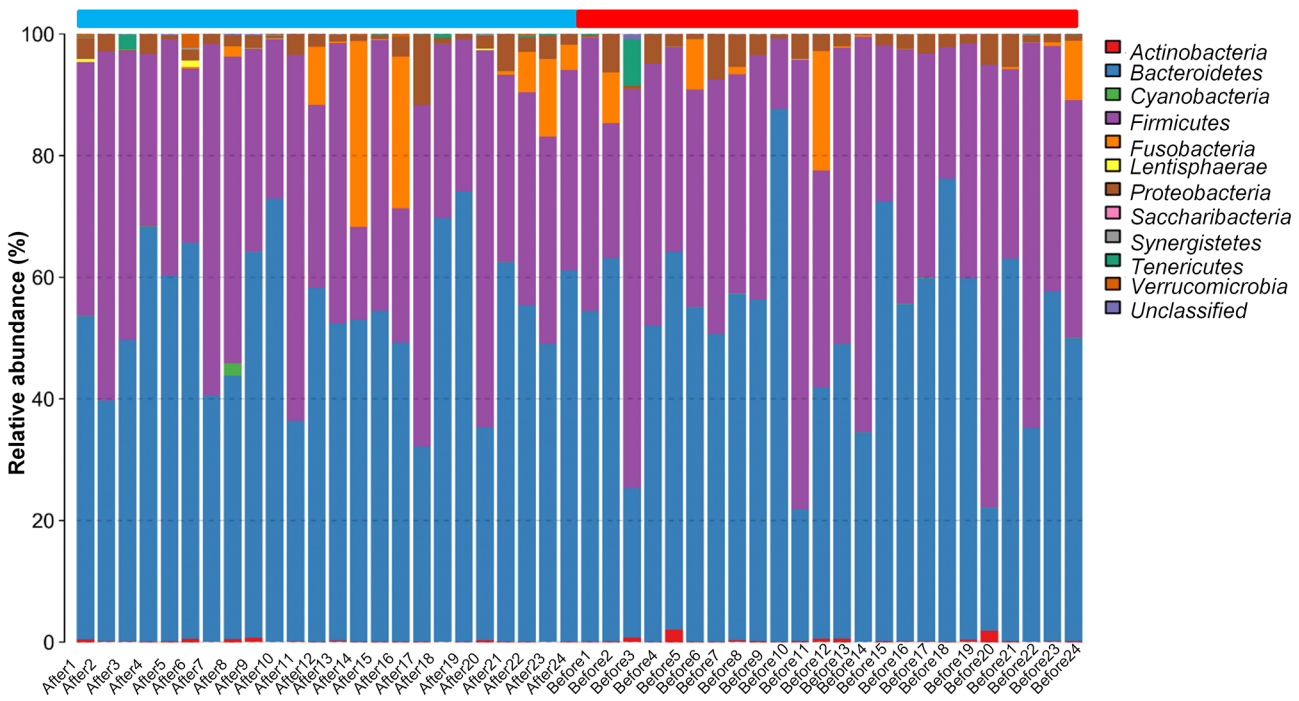

FIGURE 2 | The taxonomic composition distribution in samples at the phylum level. The abscissa represents the Sample Number. Before presents pre-intervention, After presents post-intervention. The 48 samples are divided into two parts. The blue part is from After1 to After24, and the red part is from Before1 to Before24. The legend in the upper right corner represents the name of the species at the phylum level. The content of the bacteria is displayed in the histogram in the corresponding proportion and color.

TABLE 2 | Intestinal flora diversity and abundance before and after 6-week combined exercise and diet intervention.

Alpha diversity measure	Before (mean ± SE)	After (mean ± SE)	*P*-value
Sobs	203.59 ± 10.53	240 ± 12.00	0.002
Chao	259.77 ± 14.15	299.77 ± 16.33	0.007
Ace	265.66 ± 15.33	298.70 ± 14.87	0.023
Shannon	2.93 ± 0.09	3.19 ± 0.10	0.009
Simpson	0.14 ± 0.02	0.11 ± 0.01	0.035

Sobs, observed species.

Associations of Changes in Central Hemodynamics With Changes in Gut Microbiota Composition After the Exercise and Diet Intervention

To further explore the relationship between microbial changes and cardiovascular capacity, we performed correlation analyses of cardiovascular related indicators with all microbial flora. The associations between changes in central hemodynamics and changes in gut microbiota composition are shown in **Figure 4B** and **Table S2**. Changes in SEVR, AIx75, and resting HR were positively or negatively associated with the changes in the relative abundance of 14 gut microbiota. *Akkermansia*, ambiguous taxa, *Anaerotruncus*, *Helicobacter*, *Ruminococcaceae* UCG-014, and *Victivallis* were positively correlated with SEVR, but *Cronobacter*, *Lachnospira*, *Lachnospiraceae* UCG-003, and *Prevotella* 2 were negatively correlated with SEVR. We also observed that *Cronobacter*, *Lachnospiraceae* UCG-003, and *Sutterella* had a positive correlation with AIx75, but *Helicobacter* had a negative correlation with AIx75. *Cronobacter*, *Lachnospiraceae* UCG-003, *Lachnospiraceae* UCG-010, and the *Ruminococcaceae* NK4A214 group were positively correlated with resting HR, while ambiguous taxa, *Helicobacter*, *Roseburia*, and *Ruminococcaceae* UCG-014 were negatively correlated with resting HR. *Cronobacter*, *Lachnospiraceae* UCG-003, and *Helicobacter* were all related to SEVR, AIx75, and resting HR. These findings suggested that microbial changes may be associated with cardiovascular function changes.

DISCUSSION

The present study is the first, to our knowledge, to show that the central hemodynamic changes of reduced AIx75 and resting HR and increased SEVR were associated with altered gut microbiota after a 6-week exercise and dietary intervention in adolescents with obesity.

It is known that childhood and adolescent obesity are associated with an increased risk of type 2 diabetes mellitus and cardiovascular disease in adulthood. Abnormalities in arterial and cardiac function play a critical role in the increased risk of cardiovascular events in individuals with obesity (32). It has

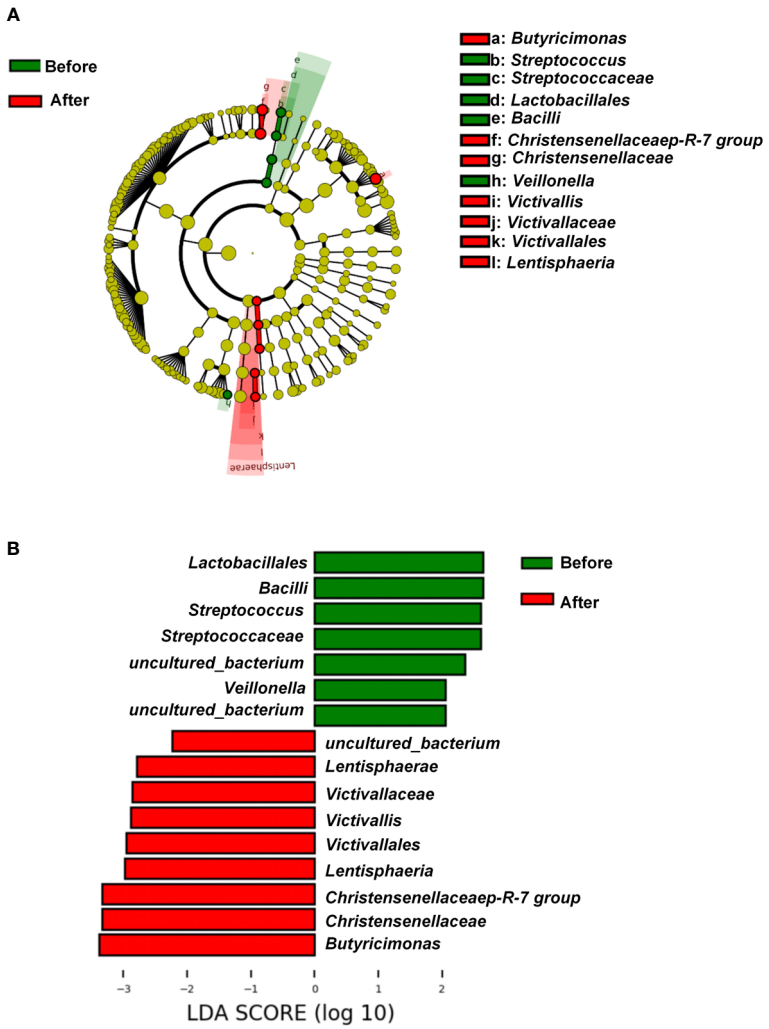

FIGURE 3 | Linear discriminant analysis effect size before and after diet and exercise intervention. **(A)** Cladogram of the gastric microbial taxa associated with individuals before and after diet and exercise intervention. Taxa higher in relative abundance before the intervention are in green, and those higher after the intervention are in red. **(B)** Histogram of the linear discriminant analysis (LDA) scores for differentially abundant taxonomic features before and after the intervention. Significance obtained by LDA effect size at $P < 0.05$, (Kruskal–Wallis test) and LDA score >2.

been established that obesity is closely related to vascular remodeling, arterial stiffness, and cardiac events (2, 33). Pulse wave analysis is a validated and noninvasive technique for estimating microvascular coronary perfusion (SEVR) and wave reflection as proxies of arterial stiffness and peripheral arteriolar resistance (AIx75). SEVR is a useful tool to evaluate cardiac workload, myocardial perfusion, and coronary flow reserve. A decrease in SEVR indicates impaired subendocardial perfusion (34). Studies by other groups have also shown that low SEVR correlates with impaired coronary flow reserve in patients with hypertension, type I and type II diabetes, or low cardiorespiratory fitness in central obesity (35, 36). SEVR has been investigated in individuals with obesity, and evolving evidence shows that SEVR is altered in children and adolescents with obesity, although the mechanism by which this effect is mediated has not been fully elucidated (16, 37). Recent studies showed that SEVR was positively correlated with insulin sensitivity in both obese and lean adolescents, suggesting an important role of SEVR in early cardiovascular risk determination in children and adolescents (38, 39). The results of the present study indicated that SEVR increased by approximately 53% in adolescents with obesity following a diet and exercise intervention program and that AIx75 was significantly decreased by approximately 22% after the intervention. Taken together, these findings suggest that adolescents with obesity had improved myocardial perfusion and vascular function after a 6-week program of exercise and diet intervention.

In recent years, with the in-depth study of intestinal flora, researchers have found that the intestinal flora is closely associated with obesity, coronary heart disease, hypertension, type 2 diabetes, cancer, and other diseases (40). The intestinal microorganisms that have been identified in

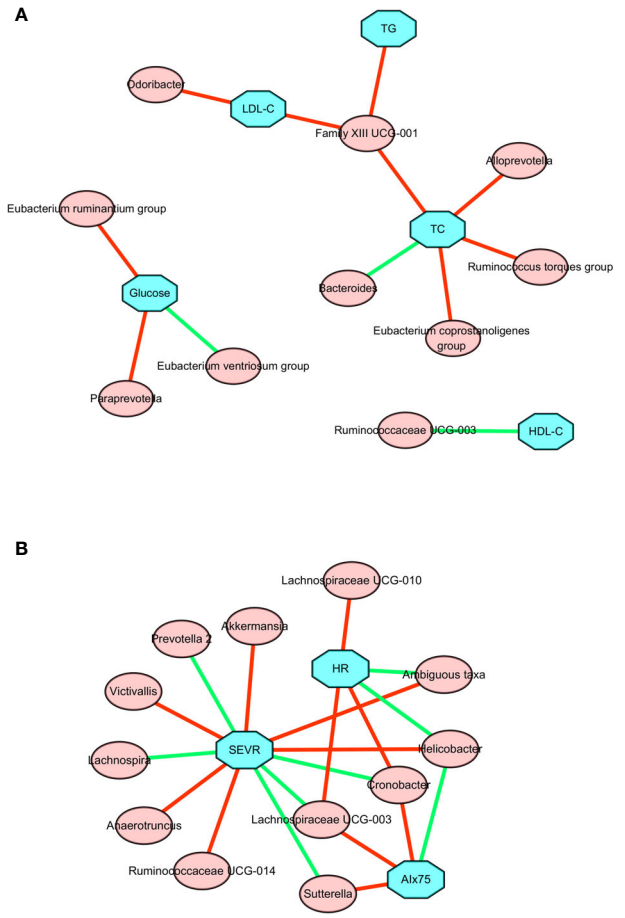

FIGURE 4 | Correlation network between changes in gut microbiota members and clinical parameters after a 6-week combined exercise and diet intervention. **(A)** Correlations between changes in gut microbiota members and metabolic parameters. **(B)** Correlations between changes in gut microbiota members and central hemodynamics. The blue square represents clinical parameters, the red circle represents gut microbiota; the red line represents the positive correlation coefficient, and the green line represents the negative correlation coefficient.

humans are mainly composed of *Bacteroides*, *Firmicutes*, *Actinomycetes*, *Proteobacteria*, and *Verrucomicrobia*. *Firmicutes* and *Bacteroides* are dominant among these gut microbiota, accounting for more than 90% of the total intestinal flora (41). There is typically a complex and delicate dynamic balance relationship between the intestinal flora and the host, and the intestinal microorganisms are rich in diversity; however, obese populations have reduced diversity (42). In our experimental intervention, a total of 956 OTUs were obtained, of which 22 were unique before the intervention, and 55 were unique after the intervention. In addition, we found that the F/B ratio was decreased after the intervention compared with that before the intervention. Some studies have suggested that *Bacteroides* and *Firmicutes* are markers of obesity, with animal models showing that the F/B ratio is higher in obese than in normal weight animals (5, 43, 44). Through our analyses of bacterial diversity and species abundance, we found that 6 weeks of exercise and diet intervention not only increased the intestinal microbial diversity of adolescents with obesity but also markedly changed the abundance of their intestinal bacterial flora. Therefore, our findings suggest that exercise and diet can change the intestinal microbial diversity and bacterial flora abundance. Although evidence has suggested F/B ratio as a potential biomarker of obesity, the value of F/B ratio varies in different populations and could be affected by many lifestyle-associated factors such as diet, physical activity, food additives and contaminants, antibiotic consumption, and physical activity (45). Therefore, the specific association between F/B ratio and the obesity needs to be elucidated in future studies.

We observed that the abundance of *Lactobacillales*, *Bacilli*, *Streptococcaceae*, and *Veillonella* was significantly reduced after the exercise and diet intervention. In a study comparing the intestinal flora of patients with obesity and coronary heart disease vs patients with obesity alone, it was found that the abundance of *Lactobacillus*, *Streptococcus*, and *Veillonella* was higher in those patients with both obesity and coronary heart disease than in those with obesity alone (46). A study of the intestinal flora of Wistar Kyoto rats (WKY rats) and WKY rats with spontaneous hypertension (SHR) found that the abundance of streptococci in SHR rats was significantly higher than that in WKY rats (47). In a study examining the intestinal flora of overweight adult women, a 6-week endurance exercise program significantly reduced the abundance of streptococci (48). In our study, adolescents with obesity did not have significant hypertension or coronary heart disease, but under experimental intervention, the abundance of *Lactobacillales*, *Streptococcaceae*, and *Veillonella* was decreased significantly. This finding suggests that our experimental intervention may effectively regulate intestinal microorganisms associated with obesity-related coronary heart disease and hypertension, thereby reducing the risk of these conditions. *Christensenellaceae* is relatively low in obese phenotypes compared with non-obese phenotypes, whereas this bacteria is enriched in lean phenotypes (49–51). Our result showed that relative abundances of *Christensenellaceae* were significantly enhanced after 6 weeks of exercise and diet intervention. In the study of obese gut flora, a variety of physiological characteristics related to obesity are affected by specific gut flora (52, 53). In our study, we found that microbial changes were significantly correlated with changes in SEVR, AIx75, and resting HR. For examples, changes in *Cronobacter*, *Lachnospiraceae* UCG-003, and *Helicobacter* were all positively or negatively associated with the changes in SEVR, AIx75, and resting HR. Such results suggest that exercise and diet interventions have the potential to improve central hemodynamics in part *via* changing the intestinal flora.

A limitation of our current study is the lack of a control group during the training program of the intervention. Since the camp was located in a remote district of the city, China, and subjects enrolled in the program came from various locations across the country and had an average BMI over 30 kg/m^2, the selection of an appropriate control group was restricted (54, 55). The lack of a designated control group allows a less clear interpretation of the

results. The results observed in this study will require confirmation in randomized controlled trials. Furthermore, due to the fact that there are only 24 participants with 15 males and 9 females, we think it is difficult to present the results by sex in the current study. Future studies on sex difference in microbiota composition will be warranted.

CONCLUSIONS

The present study showed that an exercise and diet intervention not only reduced body weight but also improved central hemodynamic measures that were associated with altered gut microbiota in adolescents with obesity. Our findings offer valuable mechanistic insights for understanding some of the effects of exercise and diet interventions on obesity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject, accession no: PRJNA675198.

ETHICS STATEMENT

This study was conducted according to the Declaration of Helsinki and was reviewed and approved by the Ethics Committee of Guangzhou Sport University (approval No. GSU2017003). The trial was registered in ClinicalTrials.gov (NCT03762629). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JH, JL, and YF carried out the experiments and analyzed the data. JH, JL, YF, HD, and HY designed a part of the study and helped to interpret part of the data. BS and MH conceived the idea, designed and supervised the study, analyzed the data, and co-wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.569085/full#supplementary-material.

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Progressive Shifts in the Gut Microbiome Reflect Prediabetes and Diabetes Development in a Treatment-Naive Mexican Cohort

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Type 2 diabetes (T2D) is a global epidemic that affects more than 8% of the world's population and is a leading cause of death in Mexico. Diet and lifestyle are known to contribute to the onset of T2D. However, the role of the gut microbiome in T2D progression remains uncertain. Associations between microbiome composition and diabetes are confounded by medication use, diet, and obesity.

Here we present data on a treatment-naive cohort of 405 Mexican individuals across varying stages of T2D severity. Associations between gut bacteria and more than 200 clinical variables revealed a defined set of bacterial genera that were consistent biomarkers of T2D prevalence and risk. Specifically, gradual increases in blood glucose levels, beta cell dysfunction, and the accumulation of measured T2D risk factors were correlated with the relative abundances of four bacterial genera. In a cohort of 25 individuals, T2D treatment—predominantly metformin—reliably returned the microbiome to the normoglycemic community state. Deep clinical characterization allowed us to broadly control for confounding variables, indicating that these microbiome patterns were independent of common T2D comorbidities, like obesity or cardiovascular disease. Our work provides the first solid evidence for a direct link between the gut microbiome and T2D in a critically high-risk population. In particular, we show that increased T2D risk is reflected in gradual changes in the gut microbiome. Whether or not these T2D-associated changes in the gut contribute to the etiology of T2D or its comorbidities remains to be seen.

Keywords: type 2 diabetes, microbiome, Mexico, metformin, deep phenotyping

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INTRODUCTION

Type 2 diabetes (T2D) is an acquired multifactorial disease that affects more than 8% of the worldwide population and leads to insulin resistance and insufficient insulin production by pancreatic islet cells (1–3). Disease onset is driven or modulated by a variety of factors such as lifestyle, diet, and genetics (4–7). T2D incidence is progressively increasing in the Mexican population and has become a major burden for the national health system and one of the leading causes of death in Mexico (8–10). The particular vulnerability of the Mexican population to this disease is driven by general factors such as a sedentary lifestyle and diet but is also influenced by genetic risk factors that are enriched in the Mexican population (11). For instance, it has been shown that about half of all native Mexicans carry an SLC16A11 variant that increases T2D risk by 20% for each haplotype (12–14). Consequently, there is an urgent need for diagnosis and treatment strategies to limit the progression of T2D in the Mexican high-risk population.

Recently, the gut microbiome has been proposed as an important modulator in the progression of T2D. Several studies have reported a wide array of associations between the gut microbiome and diabetes in European, American, and Chinese cohorts (15, 16). Most of those have suggested that the diabetic microbiome is less efficient in producing short-chain fatty acids (SCFA) due to a loss of butyrate-producing genera (17–19). However, especially when looking across different populations, the bacterial genera associated with diabetes vary (17), which is consistent with findings that the gut microbiome composition varies greatly across populations (20). For example, an increase of Proteobacteria in T2D was reported for Chinese cohorts but was absent in a European cohort (15, 16). Finding robust associations between the microbiome and T2D is further confounded by treatment effects and comorbidities. Metformin, one of the most common medications for T2D, has been shown to modify the gut microbiome which may contribute to its mechanism of action (21, 22). Indeed, studies comparing diabetic treatment-naive individuals with diabetic metformin-treated individuals showed that most of the associations initially attributed to disease progression were a consequence of the treatment and absent in individuals without a metformin treatment history (23). Apart from medication, changes in lifestyle or diet may also drive changes in the gut microbiome in a disease-independent manner (24, 25). Thus, two major treatment regimens for T2D, metformin treatment, and lifestyle intervention, will likely both trigger their own changes in the gut microbiome and need to be accounted for. Even when isolating the disease from treatment effects, associations may be confounded by comorbidities. The development of T2D is often linked with obesity, a major risk factor in the development of the disease (20, 26). Additionally, T2D increases the risk for cardiovascular disease, which itself has been linked to changes in the gut microbiome (27, 28). Controlling for all of these factors (disease treatment, lifestyle and diet, and comorbidities) might clarify the true associations between the gut microbiome and T2D disease progression. This requires deep phenotyping of the study participants where one measures not only clinically variables related to the disease of interest but also from other groups such as obesity, cardiovascular health, lifestyle, and diet. Even though this strategy has been shown to be successful in healthy individuals (29), very few studies have done so in the context of T2D.

To address these concerns and explore the relationship between the microbiome and T2D in an understudied population, we present a controlled study in a Mexican cohort from a distinct geographical region that was specifically designed to avoid those shortcomings. Except for a small control group, all participants in the study were treatment-naive and had never received prior prediabetes or diabetes diagnosis. We also combined a large array of clinical variables related to diabetes with additional phenotype measurements characterizing the lifestyle, diet, obesity prevalence, and cardiovascular health of each individual. This strategy provided a set of more than 200 clinical variables for each individual, allowing us to control for lifestyle and comorbidities and tease out associations specific to different stages of T2D progression. As a result, we identified a set of four bacterial genera that were associated consistently with T2D development. Our work establishes a set of gut microbiome markers for type 2 disease progression in a Mexican population independent of treatment effects or secondary phenotypes.

MATERIAL AND METHODS

Study Population

A cross-sectional analysis was performed in patients from Guanajuato, México, from January 2015 to December 2016, as part of the University Cohort Project CARE-In-DEEP Study (Cardiometabolic Risk Evaluation and Interdisciplinary Diabetes Education and Early Prevention). For this particular study, 470 participants who had an anthropometric, nutritional, biochemical, and metabolic evaluation, as well as a stool sample collection, were included; at the end, we had complete data and microbiome composition only for 427. Based on the oral glucose tolerance test, individuals were stratified into normal glucose metabolism (*NG*, fasting glucose less than 100 mg/dl and 2 h post-OGTT glucose less than 140 mg/dl), isolated impaired fasting glucose (*iIFG*, fasting glucose 100–125 mg/dl and 2 h post-OGTT glucose less than 140 mg/dl), isolated impaired glucose tolerance (*iIGT*, fasting glucose less than 100 mg/dl and 2 h post-OGTT glucose between 140–199 mg/dl), impaired fasting glucose plus impaired glucose tolerance (*IFG +IGT*, fasting glucose between 100–125 mg/dl and 2 h post-OGTT glucose between 140–199 mg/dl), and T2D (T2D, fasting glucose more than 125 mg/dl and/or 2 h post-OGTT glucose higher than 199 mg/dl). A survey was applied to collect general information about the use of medications, family history, risk factors, and previous diseases. The University Research Council evaluated and approved the study protocol. All participants signed informed consent.

Anthropometric Measurements

Weight was measured while participants were barefoot and wearing minimal clothing with a Tanita Scale SC-240 (Tanita

Corporation of America, USA). Height was obtained while the participants were standing barefoot with their shoulders in a normal position with a Tanita stadiometer (Tanita Corporation of America, USA). BMI (kg/m2) was obtained from standardized measurements of weight and height and was computed as the ratio of weight (kg) over height squared (m^2), defining normal weight when BMI was between 18.5–24.9 kg/m^2, overweight when BMI was between 25–29.9 kg/m2, and obesity when BMI was ≥30 kg/m^2. Waist circumference was measured at the high point of the iliac crest at the end of normal expiration to the nearest 0.1 cm. Body composition was assessed with electrical bioimpedance through a Tanita Scale SC-240. All measurements were performed by personnel trained to use standardized procedures and reproducibility was evaluated, resulting in concordance coefficients between 0.88 and 0.94.

Nutritional and Physical Activity Evaluation

A validated semi-quantitative food frequency questionnaire (FFQ) was applied to evaluate dietary intake (30). This questionnaire included data regarding the consumption of 116 food items. For each food, a commonly used portion size (e.g. one slice of bread or one cup of coffee) was specified on the FFQ and participants reported their frequency of consumption of each specific food over the previous year. Energy (kcal/day), carbohydrates (g/day), proteins (g/day), fatty acids (g/day), sucrose (g/day), and fructose (g/day) intake during the last year were obtained from this FFQ. The PA level of participants was assessed using a self-administered questionnaire that was verified when the patient assisted for the metabolic evaluation. The questionnaire has a validated Spanish translation (31), which has been adapted for use in the Mexican population. The questionnaire is self-administered and estimates the minutes devoted to the practice of different recreational physical activities during a typical week in the last year (including walking, running, cycling, aerobics, dancing, and swimming as well as playing football, volleyball, basketball, tennis, fronton, baseball, softball, and squash, among other activities). Each item includes time intervals that allow participants to detail the exact number of minutes or hours they dedicate to each form of recreational PA, as well as the intensity of each PA (light, moderate, vigorous). The total duration of each recreational PA was expressed in minutes per day. We calculated the number of hours per week devoted to each activity, which were then multiplied by the intensity of each activity, defined as multiples of the metabolic equivalent (MET) of sitting quietly. We used the Compendium of Physical Activities to assign METs to each activity (32).

Metabolic Evaluation and Oral Glucose Tolerance Test (OGTT)

All subjects were admitted to the Metabolic Research Laboratory of the Department of Medicine and Nutrition, Division of Health Sciences at the University of Guanajuato the day of the study between 7 and 8 AM, and a catheter was placed into an antecubital vein for all blood withdrawal. Subjects will not be allowed to eat or drink anything after 10 PM on the night before until the study is completed. After the intravenous catheter was placed and the first blood sample was drawn, the patients ingested 75 grams of glucose. Plasma samples for glucose measurement were drawn at -15, and 0 min and every 30 min thereafter for 2 h, glucose was measured by colorimetric glucose oxidase. Lipid levels were measured by dry chemistry with a colorimetric method (Vitros 5600; Ortho Clinical Diagnostics). According to the glucose levels at fasting and at 2 h during the OGTT, patients were classified as following: *NG* = fasting glucose <100 mg/dl and 2 h glucose <140 mg/dl, *IFG* = fasting glucose between 100–125mg/dl and a 2 h glucose <140 mg/dl, *IGT* = fasting glucose <100 mg/dl and 2 h glucose between 140–199 mg/dl, *IFG+ITG* = fasting glucose between 100–125 mg/dl and 2 h glucose between 140–199 mg/dl, *T2D* = fasting glucose >125 mg/dl and/or 2 h glucose >200 mg/dl, and *treated T2D* = previous diagnose of T2D confirmed by the medical record of the patients, consumption of hypoglycemic drugs and fasting glucose >125 mg/dl and/or 2 h glucose >200 mg/dl. HbA1c was measured according to the international guidelines by HPLC in a subset of 182 patients.

Insulin during the OGTT was measured by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (IMMULITE 1000 Siemens Healthcare Diagnostics Products Ltd). The area under the glucose and insulin curve was calculated by the trapezoidal rule.

Insulin resistance was calculated by the homeostasis model assessment (HOMA_IR) and insulin sensitivity (Matsuda Index) was derived from the insulin and glucose measurements from the OGTT as previously described (33). Insulin secretion was calculated dividing AUCinsulin_OGTT by the AUCglucose_OGTT, acute insulin response (AIR) was calculated dividing the insulin change from 0 to 30 min by the glucose change from 0 to 30 min during the OGTT; pancreatic beta cell function was estimated by the disposition index derived from the OGTT (34).

Faecal Sample Collection

Fecal samples were collected from volunteers in a sterile container, each sample was homogenized and three aliquotes placed in sterile 1 ml screw-cap tubes which were stored at -80°C before DNA extraction.

DNA Extraction

DNA extraction was performed using MoBio PowerSoil DNA Isolation kit (Mo Bio Laboratories, Inc. Carlsbad, USA) according to the manufacturer's instructions with the following modifications. After adding the C1 solution and mix, 25 µl of proteinase K solution was added and mixed by vortexing. Samples were incubated at 65°C for 10 min, during the incubation tubes were mixed by inversion every 3 min. Tubes were secured horizontally in a vortex adapter tube holder, and vortexed at 3,000 rpm for 15 min. Samples were incubated at 95°C for 10 min, during this time samples were mixed as mentioned above. Total DNA was eluted in 100 µl of sterile water. DNA concentration was quantified spectrophotometrically with a Qubit (Thermo Scientific, USA) and validated by Nanodrop (ND 2000, Thermo Scientific, USA).

16S rRNA Gene Amplification and Sequencing

DNA templates were used in a two-step PCR method to sequence the V4 hypervariable region of the bacterial 16S rRNA gene. Fusion primers contained a sequence complementary to the v4 region, as well as Nextera Illumina adapter sequences to allow multiplexing of pooled libraries.

In the initial PCR, we employing primers that were comprised of partial Nextera adapter and the V4 targeting forward or reverse primer sequence in agreement with (35).

NEXT_16S_V4_U515_F

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGG TGCCAGCMGCCGCGGTAA-3′

NEXT_16S_V4_E786_R

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGG ACTACHVGGGTWTCTAAT-3′

For each sample, we used approximately equal amounts of DNA template (up to 12.5 ng per reaction) and the reactions were carried out with a 3 min denature step at 94°C, followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. In all reactions were used 2x KAPA HiFi HotStart ReadyMix to generate the amplicons.

The amplicons were purified using Agencourt Ampure XP beads (Beckman Coulter) with a proportion of 1.25x (v/v). The PCR products were checked using electrophoresis in 2% (w/v) agarose gels in TAE buffer (Tris- acetate-EDTA) stained with SYBR Gold and visualized under UV light.

For each amplicon, a second PCR was carried out with a 3 min denature step at 95°C, followed by 8 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min with 5 ul of previous purified DNA template and using primers that attaches dual indices and Illumina sequencing adapters employing the Nextera XT kit. The PCR products were also purified equal to the first PCR reactions and the DNA concentration of each PCR product was determined using a Qubit® 2.0 Broad Range Assay (Life Technologies™). An Agilent TapeStation (Agilent, Santa Clara, CA) with DNA High Sensitivity kit was used to verify the size of the PCR product only to 23 amplicons.

All samples were random distributed in similar proportions in five pools and then mixed in equal amounts (to 10 nM). The final concentration of each pool was again determined using a Qubit® 2.0.

Pools were diluted to a concentration of 9 pM for sequencing using 2x250 bp paired-end sequencing chemistry v2 on an Illumina MiSeq platform. All samples were distributed according to the consecutive number assigned by the experimental laboratory in similar proportions in five pools and then mixed in equal amounts (to 10 nM). The final concentration of each pool was again determined using a Qubit® 2.0. Amplicons were denatured with 0.2 N NaOH and further diluted according to the MiSeq user guide, then combined with denatured PhiX control library. PhiX was spiked into the amplicon pool at 10% relative concentration. Image processing and base calling was performed on the BaseSpace cloud from Illumina (http://basespace.illumina.com).

Processing of 16S Sequencing Data

Demultiplexed MiSeq FASTQ files were analyzed using the DADA2 workflow (36). High read quality was ensured by filtering and trimming reads before further processing. In brief, the first 5' 10 bp of all reads were trimmed and reads were truncated on 3' to a maximum length of 240 and 200 bp for forward and reverse reads respectively as a dip in sequence quality was observed after that length. Furthermore, all reads with more than two expected errors under the Illumina base model were removed as well. The filtered and trimmed reads were grouped by sequencing run and the error model was fit for each run separately using the DADA2 default parameters. Sequence variants were obtained for each run separately using the previously calculated error models and the dereplicated input sequences. The sequence variants and counts were then joined across all runs in a complete sequence table and *de novo* bimera removal was run on the entire table.

Taxonomy for the final sequence variants was called using DADAs's RDP classifier and using the SILVA database (version 132) (37). Species were identified separately by exact sequence matches where possible (again using SILVA version 132). The final data set was joined with clinical metadata and saved in a phyloseq object for all downstream analyses (38).

In order to identify additional biases or batch effects. We checked whether particular sequence variant read counts were associated with DNA extraction order, DNA extraction date or the scientist that extracted the sample. We could not identify any bias visually and the distribution of correlations between the extraction date and individual sequence variant abundances was similar to one obtained from a random Poisson model. Finally, we also verified that there were no run batch effects by PCoA plots where we observed no particular separation of samples by sequencing run. a notebook for those quality control steps can be found in the study repository as described in "Data availability".

Association Tests

Association tests were run using DESeq2 with some custom adjustments (39). First, the input count matrix was filtered by a "rule of 10" where we only tested those taxa with an average count of at least 10 reads and which appeared in at least 10% of all samples. This was necessary to avoid bimodal p-value distributions during multiple testing. The count matrix was normalized across samples using the DESeq2 size factors and the "poscounts" correction for zero read counts. This ensured that associations were not driven by library size and was also expected to counteract the compositionality of the data since that normalization scheme is similar to the centered log-ratio transform (40). All continuous clinical variables were standardized (subtraction of mean and division by standard deviation). All tests used sex as a confounding variable. Age did not show major associations with any clinical variables in this study and including it as a confounder did not have any effect. Consequently, we did not include age as a default confounder in our analysis.

Association tests were then run for all combinations between taxa and clinical variables and only for those individuals with non-

missing measurements. Here, significance was evaluated based on a chi-squared likelihood-ratio test testing for a difference of deviance between the model containing only the confounder variables and a model containing the confounder variables and the tested clinical variable (39). All associations discussed in detail in this manuscript were validated manually to confirm the lack of extreme outliers in the scatter plots. P-values were adjusted for false discovery rate using independent hypothesis weighting to avoid biases for tests with low abundance taxa (41).

RESULTS

The Microbiome of Treatment-Naive Individuals Associates With a Wide Range of Clinical Variables

We recruited a cohort of treatment-naive subjects from the Guanajuato region of Mexico as part of the CARE-In-DEEP Study (Cardiometabolic Risk Evaluation and Interdisciplinary Diabetes Education and Early Prevention) of the University of Guanajuato. This cohort consisted of 405 individuals with no previous diabetes diagnosis and a control group of 25 subjects with previously diagnosed T2D or a history of metformin treatment (see **Figure 1A**). Each of the participants in the study underwent extensive clinical characterization consisting of direct measurements as well as a set of validated questionnaires, forming a data set of 226 clinical variables spanning the areas of diabetes, obesity, general health, lifestyle, and diet (**Figure 1B**). Based on an oral-glucose tolerance test, subjects were stratified into five metabolic groups ranging from normoglycemia and normal glucose tolerance (NG, n = 214), to different types of prediabetes (impaired fasting glucose, IFG n = 52, impaired glucose tolerance IGT n = 42, and IFG+IGT n = 57), and T2D (new T2D n = 48, and treated previous T2D n = 17) (see Methods and **Figure 1C**). As shown in **Table 1**, clinical phenotype varied widely between metabolic groups, with a progressive increase in weight, body fat, glycated hemoglobin (HbA1c), glucose levels, and deteriorating insulin sensitivity and pancreatic beta cell function from the NG group to the T2D group (see **Figures S1A–C**). **Table 2** also shows the frequency of T2D risk factors between the study groups. As shown, only age >45 years, overweight, dyslipidemia, and high blood pressure were significantly different between groups.

To identify links between the microbiome and the progression of T2D, we sequenced the 16S rDNA V4 amplicon from stool samples of the cohort. Sequencing data was analyzed using DADA2 which identified 17,059 exact amplicon sequence variants across all samples (see Methods). These sequence variants mapped to 378 bacterial genera, however only 629 sequence variants and 125 genera were appreciably frequent across samples (found in >10% of individuals).

Previous studies have found metformin treatment to lower *Intestinibacter* abundances and to increase Escherichia abundances (23). We found similar trends in our data, albeit not significant (Mann-Whitney p = 0.05 and 0.07 for *Instestinibacter* and *Escherichia*, see **Figure S1D**). In general, T2D could only be weakly predicted from microbiome composition (Random Forest area under ROC = 0.69, see **Figure 1D**).

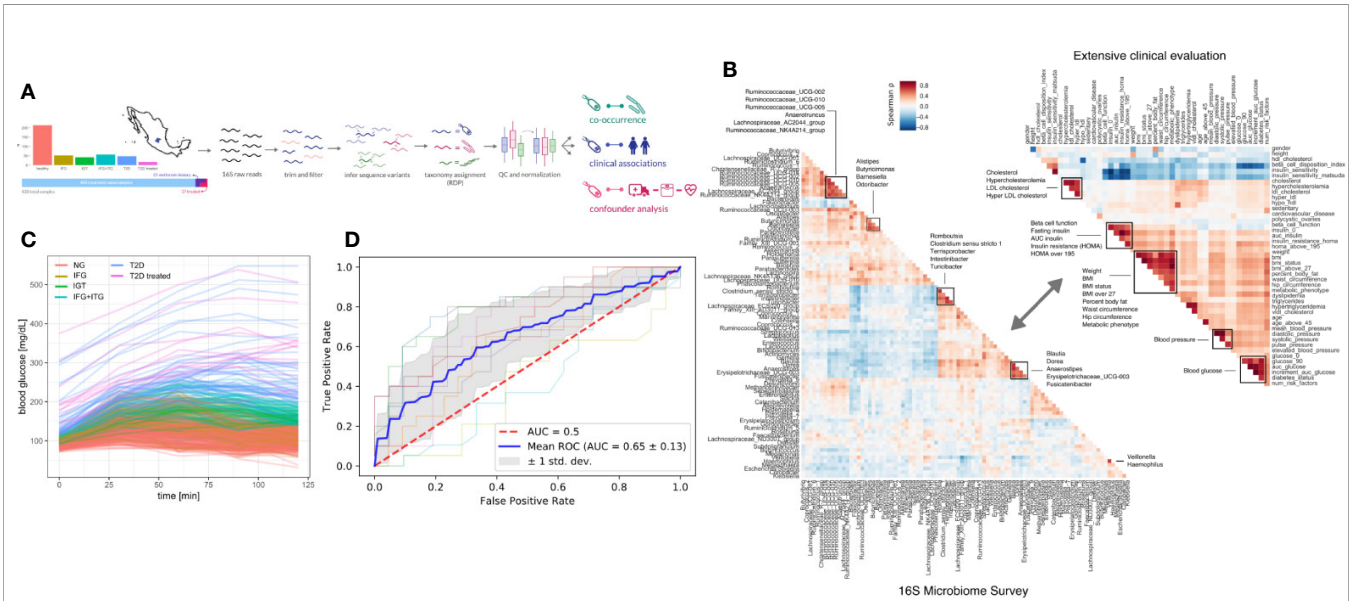

FIGURE 1 | Study design. **(A)** 405 Individuals were recruited from Guanajuato state and classified into normoglycemic (NG), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), impaired fasting glucose, and impaired glucose tolerance (IFG+IGT), and type 2 diabetes (T2D). Twenty-five individuals under treatment for a previous T2D diagnosis or with previous metformin history were added as controls (T2D treated). **(B)** Correlations (Spearman ρ) between bacterial genera in the study (intra-microbiome) are shown in the left correlation matrix whereas correlations between clinical variables are shown in the right correlation matrix. **(C)** Blood glucose curves for all individuals in the study colored by classification. **(D)** Receiver-Operator curves for predictions from a Random Forest model. Individual cross-validation curves are shown along with the mean trend and standard deviations.

TABLE 1 | Cohort characteristics.

Variable (n = 430)	NG (n = 214)	IFG (n = 52)	IGT (n = 42)	IFG+IGT (n = 57)	new T2D (n = 48)	treated T2D (n = 17)	p value
Age (y)	38 ± 14	45 ± 14[a]	45 ± 12	49 ± 12[a]	51 ± 9[a]	55 ± 11[a]	<0.001
Sex (M/F)	49/165	23/29	13/29	18/39	17/31	2/15	0.019
PP (mmHg)	38 ± 9	41 ± 10	42 ± 8	45 ± 11[a]	45 ± 12[a]	44 ± 12	<0.001
Weight (kg)	70 ± 15	77 ± 17	76 ± 15	85 ± 17[a]	85 ± 20[a]	74 ± 23	<0.001
BMI	26.5 ± 5.4	28.2 ± 5.1	29.3 ± 5.3[a]	32.4 ± 6.2[ab]	32.5 ± 7.0[ab]	31.0 ± 9.0[a]	<0.001
Body fat%	33 ± 8	32 ± 7	35 ± 8	39 ± 8[ab]	39 ± 10[ab]	37 ± 9	<0.001
Visceral fat %	6.6 ± 4.1	9.3 ± 3.9[a]	9.3 ± 3.9[a]	11.8 ± 4.3[a]	12.2 ± 5.6[a]	10.3 ± 4.2	<0.001
WC (cm)	84.7 ± 13.4	90.3 ± 11.2	92.0 ± 10.3[a]	98.4 ± 13.3[a]	97.7 ± 18.5[a]	95.4 ± 10.5	<0.001
Glucose (mg/dl)	88 ± 7	105 ± 4[a]	94 ± 4	108 ± 5[a]	154 ± 60[abcd]	190 ± 74[abcde]	<0.001
HbA1c %	5.3 ± 0.3	5.4 ± 0.3	5.4 ± 0.4	5.6 ± 0.4	6.6 ± 1.6[abcd]	7.5 ± 1.2[abcd]	<0.001
Total cholesterol (mg/dl)	181 ± 37	189 ± 36	190 ± 32	196 ± 42	188 ± 30	218 ± 30[a]	<0.001
TG (mg/dl)	131 ± 62	173 ± 93[a]	171 ± 78[a]	181 ± 80[a]	200 ± 85[a]	202 ± 66[a]	<0.001

P value column denotes p values of ANOVA with Bonferroni correction. Superscript letters denote the following: (a) p < 0.01 vs NG (b) p < 0.01 vs IFG (c) p < 0.01 vs IGT (d) p < 0.01 vs IFG +IGT (e) p < 0.01 vs treatment-naive T2D.

TABLE 2 | T2D risk factors between the study groups.

(n = 430)	NG (n = 214)	IFG (n = 52)	IGT (n = 42)	IFG+IGT (n = 57)	new T2D (n = 48)	treated T2D (n = 17)	p value
T2D risk factors (%)							
Physically inactive	61.2	59.6	80.0	71.9	66.0	64.7	0.211
Age >45y	39.2	57.7	47.5	68.4	80.9	76.5	<0.001
T2D Family history	73.7	75.0	77.5	80.7	83.0	100.0	0.160
Overweight	41.8	50.0	64.3	80.7	81.3	64.7	<0.001
Dyslipidemia	69.7	84.6	85.7	89.5	87.2	100.0	<0.001
HBP	25.2	46.2	45.2	54.4	64.6	58.8	<0.001

HBP, high blood pressure.

We identified potential links between the microbiome by exhaustive testing of all combinations between bacterial genera and clinical variables, including alpha diversity (Shannon index). This required careful modeling of the sequencing counts which often do not follow normal distributions. Here we chose negative binomial models as they model the high prevalence of zero read counts and have been shown to represent amplicon sequencing data well (42). Consequently, associations between the microbiome and clinical variables were identified by a robust normalization and testing strategy based on DESeq2 (see Materials and Methods). In summary, read abundances were normalized for library size, regressed against clinical variables with negative binomial models, and significance was judged by a chi-squared likelihood-ratio test on model deviances (LRT) (39). Of the 30,780 tests, 208 were deemed significant under an FDR cutoff of 0.05 (**Figures 2A, B**). Clinical measurements related to obesity had the most significant associations with microbiome features, while diet-related variables were the least likely to yield a significant association (**Figure 2A**). The relative paucity of associations between the microbiome and diet may be a consequence of the homogeneous geographical location and the long time-frame covered by the food questionnaire. Additionally, dietary changes usually induce large short-lived shifts in the microbiome which are commonly studied using beta-diversity (43). Those global changes may affect many genera which may conflict with the normalization method used here that required the majority of taxa to be non-differential across individuals.

The genera associated with the most clinical variables was the facultative anaerobe *Escherichia* and the obligate anaerobe *Veillonella*, which had 36 and 23 significant associations respectively (**Figure 2B**). *Escherichia* was associated mostly with variables related to diabetes and obesity whereas *Veillonella* was associated with variables from many categories. *Ruminococcaceae* genera were the most positively correlated with alpha diversity (Shannon index) whereas *Fusobacterium*, *Flavonifractor*, and *Parasutterella* were the most negatively associated with alpha diversity (Shannon index, **Figure 2C**).

The gut microbiome of the treatment-naive cohort was associated widely with T2D-related clinical variables. A set of 14 bacterial genera associated at least weakly with 25 of the 31 diabetes-related measures (FDR-corrected LRT p-value < 0.05). However, we observed large differences in how those associations distributed across genera (**Figure 3A**). Whereas some genera associated with a wide array of T2D measures (for instance *Escherichia*) other associated only with a single measure (e. g. *Ezakiella* with T2D family history), or exclusively with glucose-related measures, but not insulin-related measures (e. g. *Romboutsia*, **Figure 3A**). In general, we observed more associations with glucose metabolism than insulin levels. *Escherichia* showed by far the most associations with T2D measures and notably associated with all glucose measures included in the study. Given the observed genus-specific patterns of association with T2D, this raised the question of how one could identify a subset of genera that were consistent markers of overall disease progression.

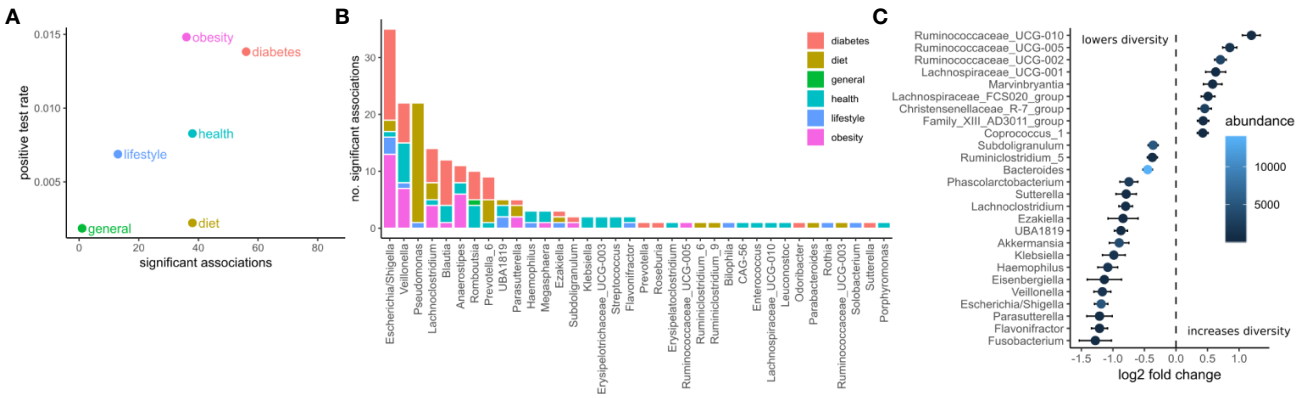

FIGURE 2 | Associations between the microbiome and phenotype. **(A)** The number of significant associations between the microbiome and clinical variables grouped by category (FDR corrected LRT $p < 0.05$). The positive test rate denotes the significant tests/total tests for the category. **(B)** Significant tests per genus (FDR corrected LRT $p < 0.05$). Color denotes the category of the clinical variables the genus associates with. **(C)** Significant associations (FDR corrected LRT $p < 0.05$) between bacterial genera and alpha diversity (Shannon). Points denote the log fold change (DESeq2 regression coefficient) of a genus when the diversity increases by one standard deviation. Error bars denote the standard error of the coefficient. Fill color denotes the mean of normalized reads across all samples.

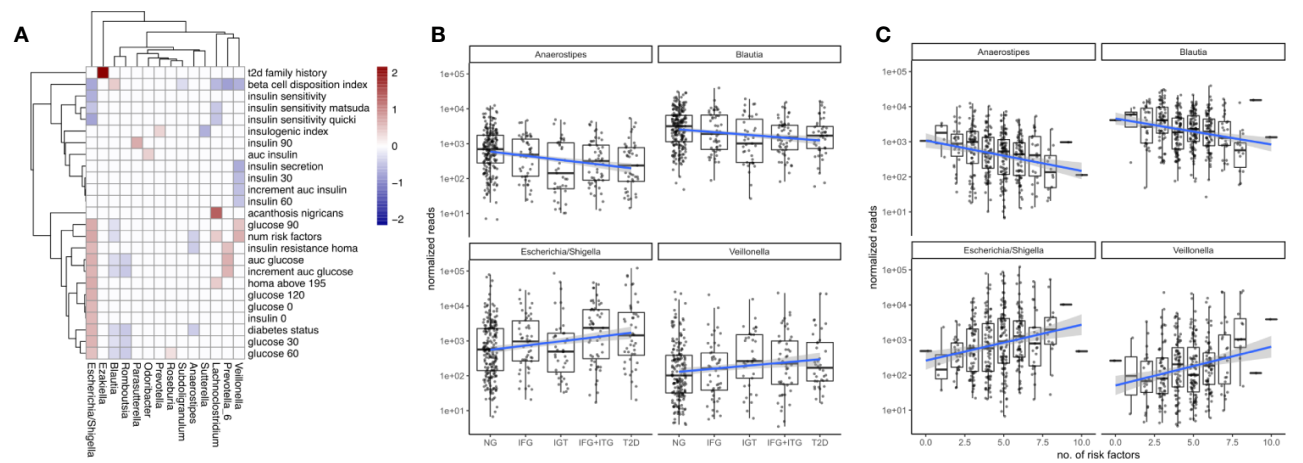

FIGURE 3 | Associations between microbiome composition and disease progression. **(A)** Significant associations (FDR corrected LRT $p < 0.05$) between bacterial genera and T2D clinical variables. White boxes denote a lack of significant associations ($p > 0.05$) and fill denotes regression coefficient between genus and variable (log2 fold change in genus abundance if the variable is increased by one standard deviation). **(B)** Associations between disease state and selected bacterial genera. Blue lines indicate regression lines and light gray bands denote the standard error of the regression. **(C)** Overall T2D risk was evaluated by the number of T2D risk factors associated with the same genera as observed earlier. This relationship was gradual across the number of risk factors.

A Group of Distinct Bacteria Mark the Gradual Progression of Type 2 Diabetes and Modulate Persistent Inflammation

To identify bacterial genera that were strong markers for disease progression, we asked whether some of the 18 genera associating with diabetes measures would do so in a gradual manner across disease progression and risk. Disease progression was quantified by ordering the metabolic groups by severity ranging from normoglycemic (NG) to fully developed T2D. Disease risk was assessed by a set of manually chosen binary indicators (absent/present) for known risk factors and counting their occurrences for each individual (see Materials and **Figure S2**). Thus, an individual with 8 risk factors would be considered at higher general risk for developing T2D than an individual with only 2 risk factors. Metabolic groups and the number of risk factors did only moderately correlate with each other (Spearman rho = 0.45), confirming that they described different aspects of the disease. Treating the metabolic groups as well as the number of risk factors as continuous descriptors we identified a set of 4 bacterial genera that associated at least weakly with both of them (*Escherichia*, *Veillonella*, *Blautia* and *Anaerostipes*, FDR-corrected LRT $p < 0.1$). It should be noted that those gradual changes do not reflect longitudinal changes within individuals but rather continuous associations with severity across the entire population.

We found that *Escherichia* and *Veillonella* were positively associated with the diabetic state, increasing in abundance with disease progression from normal to T2D (**Figure 3B**). Conversely, *Blautia* and *Anaerostipes* abundances declined with disease progression (**Figure 3B**). Whereas *Escherichia* and *Veillonella* are both associated negatively with alpha diversity (Shannon index), *Anaerostipes* and *Blautia* did not (compare **Figure 2C**). Therefore, the protective association between these genera and T2D cannot be explained by an increased diversity alone. Intriguingly, more than 99% of the *Anaerostipes* sequence variants with unique species assignments belonged to the species *Anaerostipes hadrus*, a known butyrate producer. The four identified genera showed a continuously increasing or decreasing trend with disease progression, with only the prediabetes group (IGT) showing some deviation from this trend (**Figure 3B**).

For all of the identified genera, the number of risk factors aligned linearly with the log-transformed counts. Median *Escherichia* levels increased by almost 2 orders of magnitude between individuals with 2 and 8 risk factors respectively and *Anaerostipes* decreased by one order of magnitude (**Figure 3C**). Notably, individual binary risk factors did show only very few associations with the identified genera (**Figure S2**). Thus, the accumulation of T2D risk factors across the entire cohort, including healthy individuals, is gradually linked to changes in the microbiome.

All of the 4 presented genera were also associated with the primary clinical indicators for T2D. Higher levels of *Escherichia* and *Veillonella* accompanied higher area under the glucose curve and diminished beta cell function (FDR adjusted LRT $p < 0.05$, see **Figure 4**). However, *Escherichia* was the only genus that was significantly associated with glycated hemoglobin (log2 fold change 0.5, FDR-adjusted LRT $p = 0.04$), and insulin sensitivity (Matsuda index, FDR adjusted LRT p = 7e-5). Higher levels of *Blautia* and *Anaerostipes* on the other hand were associated with lower areas under the glucose curve and normal beta cell function (FDR adjusted LRT $p < 0.05$, see **Figure 4**). Thus, the associations with markers of metabolic health were consistent with the results of oral glucose tolerance tests.

We then asked whether the patterns of these four microbiome markers of the disease might be reversed by treatment. In a control group of subjects that had already received T2D treatment, we noted that type 2 diabetes treatment (mostly metformin alone or in combination with other drugs) led to an approximate return of the 4 genera to normal levels (Mann-Whitney test p values between 0.4–0.9, see **Figure 4C**). This behavior was not observed for all genera. For instance, Romboutsia levels were not affected as strongly by diabetes treatment (Mann-Whitney test $p = 0.07$, **Figure 4C**). Thus, anti-hyperglycemic treatment for glucose control was sufficient to return the identified genera close to normal levels and this was not the case for other bacterial genera.

Because invasion with Proteobacteria such as *E. coli* is often a sign of intestinal inflammation we also investigated the association with the identified taxa with inflammation markers. We found that *Veillonella* increased with higher concentrations of C-reactive protein (CRP) whereas *Blautia* and *Anaerostipes* decreased with higher concentrations of interleukin 6 (IL-6) in treatment-naive individuals (all FDR-corrected LRT $p < 0.1$, **Figures 5A, B**). Strikingly, both of the identified inflammation markers were increased in treatment-naive diabetic individuals compared to healthy individuals and remained elevated in treated individuals (**Figures 5C, D**). Thus, in contrast to microbial shifts, the increase in inflammation markers is not ameliorated by T2D treatment.

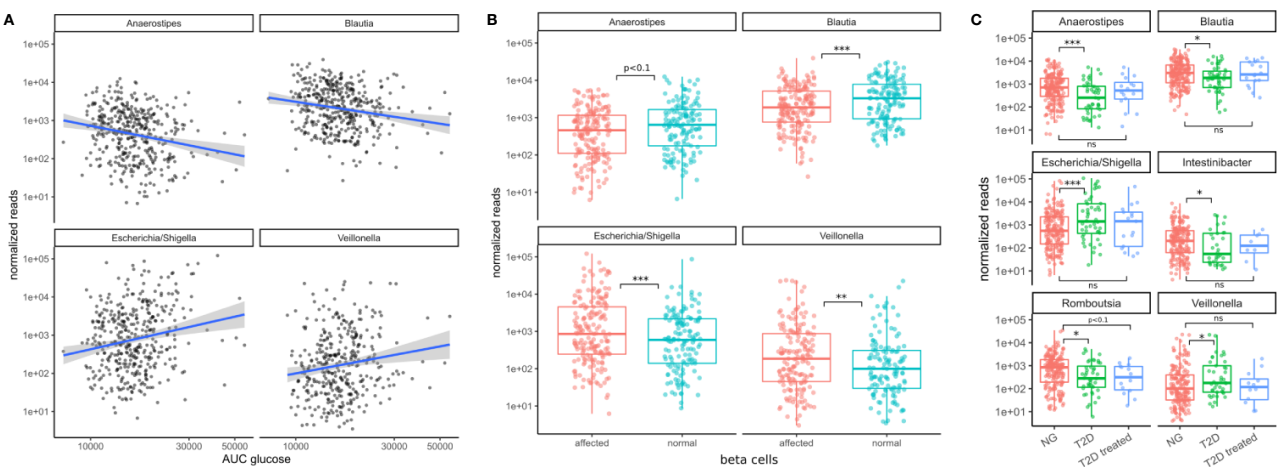

FIGURE 4 | Associations between bacterial genera and the primary T2D-related clinical measurements. **(A)** The identified genera associated with the area under the glucose curve (AUC glucose). AUC values were rank-transformed in the panel to make the regression independent of outliers. The blue line denotes a linear model between log-transformed normalized counts and rank transformed AUC values. **(B)** Bacterial abundances stratified by beta cell function ("affected" meaning beta cell function was negatively affected). Normal beta cell function was identified by a beta cell disposition index larger than 2 (see **Figure S1B**). Stars denote significance under the likelihood ratio test. **(C)** T2D treatment restored some of the altered bacterial genera (*Anaerostipes*, *Blautia*, *Escherichia*, *Veillonella*) to their normal levels but this was not true for all of them (Romboutsia remained at low levels). Stars denote significance under a Mann-Whitney test. For B–C "*" denotes $p < 0.05$, "**" denotes $p < 0.01$, and "***" denotes $p < 0.001$.

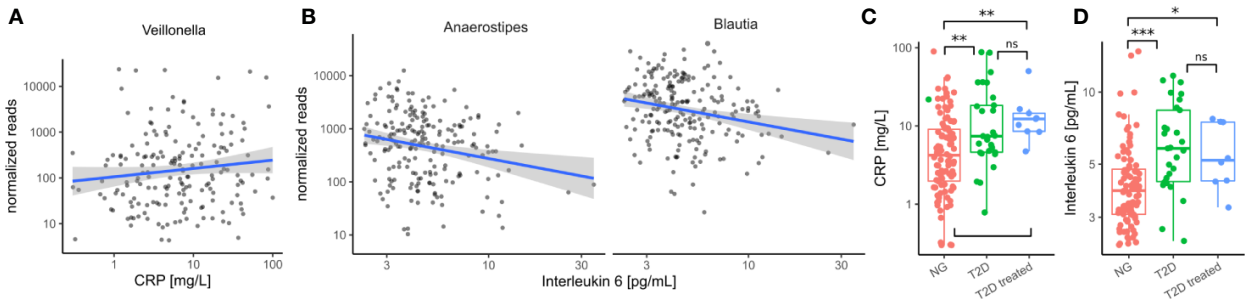

FIGURE 5 | Immune/Inflammation makers in the cohort. **(A)** Association between *Veillonella* read counts and C-reactive protein in treatment-naive individuals. (CRP). **(B)** Association between *Anaerostipes/Blautia* read counts and Interleukin 6 in treatment-naive individuals. **(C)** C-reactive protein levels were elevated in untreated diabetic individuals (T2D) and this was not altered by treatment. **(D)** Interleukin 6 levels were elevated in untreated diabetic individuals and this was not altered by treatment. In C–D Stars denote significance under a Mann-Whitney test (* - $p < 0.05$, ** - $p < 0.01$, *** - $p<0.001$).

A Confounder Analysis Across Variable Classes Identifies Diabetes-Specific Associations

As mentioned before, T2D shows comorbidity with many other clinical conditions such as obesity and cardiovascular disease. For instance, we observed correlations of the major glucose metabolism measurements such as the area under the glucose curve and insulin sensitivity with obesity-related variables such as BMI, visceral fat, and waist-to-hip ratio (see **Figure 1D**). Thus, there was a possibility that our observed changes across disease progression were driven by other covariates. For instance, the association between a bacterial genus and glucose metabolism may be a consequence of obesity which itself is associated with higher glucose levels. This is commonly known as confounding and obesity would be the confounder in that case.

To assess those putative confounding effects, we selected three groups of primary clinical variables that were available for the majority of the samples for T2D, obesity, and cardiovascular health, respectively (see Materials and Methods). Representative clinical variables were chosen by considering only variables measured for the majority of individuals (not all individuals provided information on all measures) and that showed the strongest association with bacterial abundances by themselves. For each of the previously identified bacterial genera and each variable in the three groups, we then ran association tests with either only sex as the confounder ("without") or with sex and all major variables from the other groups as confounders ("with"). The strength of confounding was evaluated by looking for changes in the regression coefficient for the association between bacterial abundance and the respective clinical variable. If the coefficients were stable across the non-confounded ("without") and ("with") group we judged the association robust, whereas a coefficient closer to zero in the confounded setting ("with") would indicate a diminished association when correcting for additional covariates and, thus, a spurious association.

Coefficients for the diabetes-related clinical variables were not significantly impacted by the introduction of the additional confounders (see **Figure 6**), whereas the coefficients for obesity-related variables were almost completely abolished by adding the additional confounders. This means that the associations between the four identified bacterial genera and obesity-related clinical variables were essentially lost when correcting for diabetes status. Thus, diabetes measures explained most of the associations between bacterial abundances and obesity but not *vice versa*. Cardiovascular health was also confounded heavily by the T2D-related variables. In particular, we observed that association coefficients between the tested microbial genera and BMI, body fat, or diastolic pressure changed sign when correcting for secondary clinical variables (**Figure 6**). This indicates that non-corrected associations can misinterpret the isolated effect of those clinical variables. Those spurious associations with obesity or cardiovascular disease could be observed with all of the four genera identified in our previous analysis. Here, only *Veillonella* showed residual associations with body fat and blood pressure after correction for some of the clinical variables (body fat and blood pressure) which led us to hypothesize that *Veillonella* seems to associate unspecifically with a variety of "bad health" markers.

DISCUSSION

One of the challenges in studying the connections between the gut microbiome and T2D is the strong effect of medication on the gut microbiota. Metformin in particular has been shown to induce changes in the microbiome that may themselves alleviate some of the symptoms of T2D either directly or indirectly (22). Consequently, T2D medication with metformin may mask T2D-specific changes in microbial composition. We confirm this in our study and avoided those treatment-specific effects by concentrating on a large treatment-naive cohort. This allowed us to identify a set of four bacterial genera that are closely connected to T2D disease progression and risk in treatment-naive individuals of a high-risk population. Notably, all of the four identified genera returned to near-normal levels in treated individuals. Thus, we found that metformin does not only affect more taxa in the

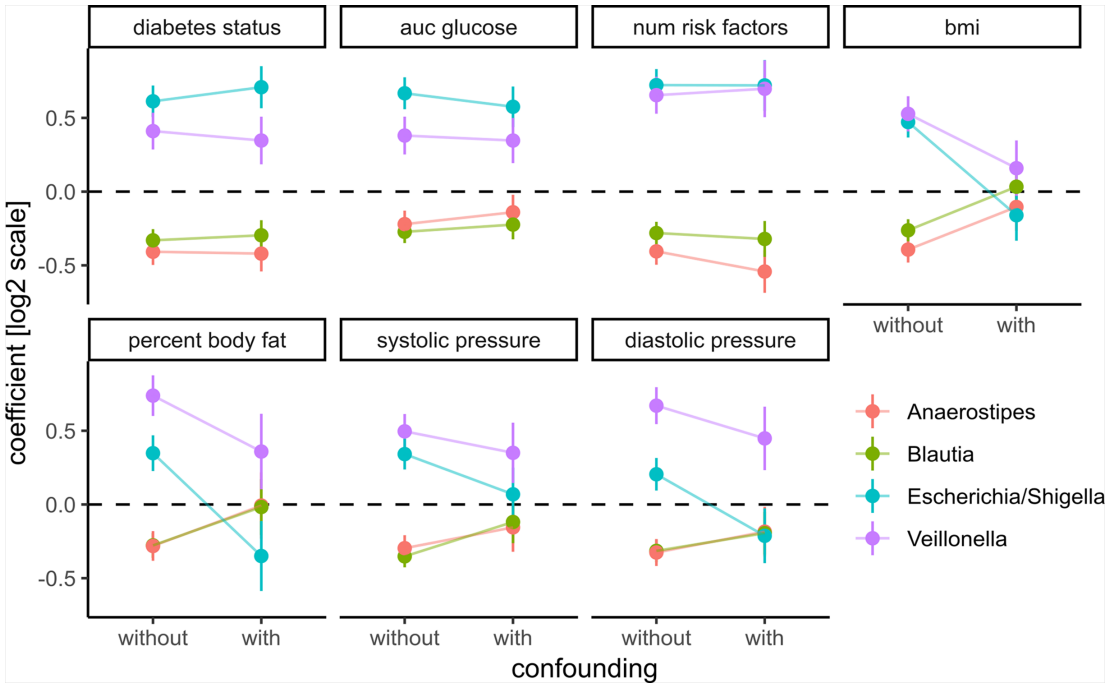

FIGURE 6 | Adding prominent confounders from other classes of clinical variables did not influence effect size for diabetes-related clinical response variables but did abolish associations in obesity and some cardiovascular responses. Clinical variables are grouped into T2D, obesity, and cardiovascular disease, and association tests between each bacterial genus and variable are either not confounded with additional variables (without confounding) or confounded with all variables from the other groups (with confounding). Points denote the coefficient associated with the response variables under the DESeq2 model (log fold change associated with an increase of one standard deviation in the clinical variable) and error bars denote the standard errors of the model coefficient. Colors denote bacterial genera.

gut microbiome than suggested previously but may also completely disguise microbial changes induced by T2D (**Figure 4A**). It is unclear whether this medication-induced restoration of the gut microbiome is a consequence of alleviated symptoms such as the regulation of blood glucose levels or a direct interaction between drugs and the microbiome. However, our observation that metformin treatment counteracts microbial changes associated with T2D but not with other bacteria seems to suggest that this happens in a disease-dependent manner. Importantly, treatment did not lower the concentrations of the microbiome-associated inflammation markers CRP and IL-6 which indicates that there may be secondary effects of T2D that persist after treatment.

Additionally, the inclusion of a complete characterization of individual phenotypes uncovered the complex pattern of connections between microbial taxa and T2D. Most (25/31) of the diabetes-related covariates included in the study did associate with at least one microbial taxon. However, individual taxa would usually associate with a specific set of clinical measurements. For instance, even though *Escherichia* and *Veillonella* both increased with disease progression, *Escherichia* was preferably associated with measures of blood glucose whereas *Veillonella* was associated with more insulin-related measures (**Figure 3A**). Additionally, we also found that *Blautia* and *Anaerostipes* did not only decrease with disease progression but also associated with improved beta cell function and insulin efficiency, which is to our knowledge the first time this connection has been described.

We also studied the relationship between the identified bacterial genera and T2D risk based on several established T2D risk factors. Here, we found a clear pattern of microbial shift associated with the accumulation of risk factors. This complements previous studies that have described a connection between the microbiome and the coincidence of T2D diabetes but not on T2D risk itself (23). We observed that this association was stable even in a subpopulation with a low number of risk factors. This is consistent with the pathophysiology of T2D and shows that T2D-specific changes in the microbiome may precede observable symptoms (44, 45). At the present point, one cannot say whether those associations observed across our cohort are indeed present in single individuals during the disease trajectory. Thus, those results rather present co-occurrence than causality. Longitudinal studies may capture properties of T2D progression that are missed by cross-sectional studies (46). Thus, we anticipate that future longitudinal studies will shed more light on the causality between diabetes and the microbiota.

Nevertheless, deep clinical phenotyping allowed us to control for many of the known comorbidities of T2D and confirm the robustness of our findings. For instance, we show that the

strongest associations between the microbiome and obesity-related clinical indicators (BMI and visceral fat) are almost entirely confounded by diabetes covariates and cannot be maintained when controlling for diabetes status. The implications of this observation go beyond this study and demonstrate the potential for extensive confounding in microbiome-obesity studies. As we have shown, this can be avoided by extensive phenotyping of the study subjects and can help to identify effects that are specific to the studied condition and not a secondary effect of another phenotype. In particular, we feel that the combination of correcting for additional phenotypes combined with studying microbial changes that are reversed by treatment is a feasible strategy to constrain the number of associations and identify connections between disease and the microbiome that are good candidates for causal relationships.

On a coarse level, our study is in agreement with previous T2D microbiome studies which mostly report a depletion of butyrate producers. On a fine level, however, we find that the identified genera in our study differ from what has been found in previous studies. For instance, we do not find a depletion of the butyrate-producing *Roseburia*, *Faecalibacterium*, or *Eubacterium* (18) but rather observe a decrease in *Anaerostipes hadrus*, another known butyrate-producer (47). Some studies have also reported an increase of *E. coli* (15, 16), however, we do not observe an increase in *Lactobacillus* or *Streptococcus* as reported there. Consistent with previous findings in treatment-naive subpopulations, we found that T2D could only be weakly predicted from microbiome composition when correcting for metformin treatment (23). Hyperglycemia itself has been shown to increase the risk for enteric infection by driving intestinal barrier permeability which is consistent with the tight association we observe between *Escherichia* abundance and blood glucose levels (48). Functionally, many of the observed associations point towards gut inflammation. Blooms of proteobacteria, like *E. coli*, have been associated previously with an inflamed gut and are often observed in irritable bowel disease (49, 50). Loss of *Blautia* has also been associated with an inflamed gut in Crohn's disease and other clinical conditions (51, 52). Additionally, alterations in solute carrier expression, as present in the Mexican population (12), have been observed in the development of irritable bowel disease and have been linked to inflammation (53, 54).

Though there is some evidence that gut inflammation may be modulated by the microbiome, it is still unclear whether one could potentially target T2D *via* altering the gut microbiome (55, 56). We did identify a microbiome-inflammation axis formed by three of the four identified taxa and observed that inflammation markers remained elevated even in treated individuals. *E. coli* was not directly associated with CRP or IL-6 (FDR-adjusted LRT $p = 0.7$) which indicates that there may be additional factors driving the colonization with Proteobacteria. Thus, the observed compositional changes consistent with inflammation might be useful as markers for long-term effects of diabetes-induced phenotypes. For instance, the gut microbiome may help to identify diabetes patients with a high risk for irritable bowel disease or colorectal cancer which have a higher incidence in T2D patients (57–59). In the end, additional studies will be required to elucidate the causal connections between the gut microbiome and T2D.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. Raw sequencing data along with metadata is provided on the sequence read archive (SRA) under the Bioproject PRJNA541332 at https://www.ncbi.nlm.nih.gov/. All additional primary input files as well as intermediate files and R notebooks to reproduce the analysis and figures are provided at https://github.com/resendislab/mext2d. More complex functions that could be potentially useful in the analysis of other data sets are furthermore provided along with documentation in a dedicated R package at https://github.com/resendislab/mbtools.

ETHICS STATEMENT

The study protocol was reviewed and approved by the Research Council of the University ofGuanajuato. All participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OR-A, RG-M, and EA designed the study and wrote the manuscript. CD analyzed the data and wrote the manuscript. MR-E, LJ-C, MM, and CG-N extracted the samples and obtained the clinical data. NC and VZ performed the co-occurrence and machine learning analyses and wrote the manuscript. MT analyzed the diet data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.602326/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | (A) Increment of the area under the glucose curve stratified by metabolic group. "nT2D" denotes new (treatment-naive) T2D and pT2D denotes previous (treated) T2D. Bars denote the standard error of the mean. **(B)** Insulin sensitivity over the metabolic groups. Bars denote the standard error of the mean. **(C)** Beta cell disposition index stratified by metabolic group. The dashed line denotes the cutoff value 2 that was used to separate functional from non-functional beta cells. **(D)** Abundances of Escherichia and *Intestinibacter* stratified by metformin history. Superscripts denote the following comparisons in a t-test: *$p < 0.01$ vs. NG group, **$p < 0.01$ vs. NG and IFG groups, $^{\dagger}p < 0.01$ vs all groups.

SUPPLEMENTARY FIGURE 2 | Binary risk factors used to calculate the overall risk of developing T2D. Shown are bacterial abundances stratified by individual risk factors. Absence is denoted by zero and presence by 1. The following 3 risk factors were summarized into a single one (any of the 3 present) when calculating overall risk: polycystic ovary syndrome, cardiovascular disease, having a baby born with more than 4 kg of weight (macrosomia). Reads were normalized across samples as described in Methods.

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The Impact of Gut Microbiome on Metabolic Disorders During Catch-Up Growth in Small-for-Gestational-Age

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Objective: Catch-up growth (CUG) in small for gestational age (SGA) leads to increased risk of metabolic syndrome and cardiovascular diseases in adults. It remains unclear if microbiota could play an important role in CUG-SGA independent of genetic or nutritional factors. The present study explored the role of gut microbiota in, and its association with, metabolic disorders during CUG-SGA.

Methods: An SGA rat model was established by restricting food intake during pregnancy, and the rats were divided into catch-up growth (CUG-SGA) and non-catch-up growth (NCUG-SGA) groups based on body weight and length at the fourth postnatal week. High-throughput sequencing of 16S rRNA was conducted to detect the diversity and composition of the gut microbiota. Fecal short-chain fatty acids (SCFAs) were detected by gas chromatography-mass spectrometry. Transcriptome sequencing of liver tissue was performed and verified using real-time PCR. Concentrations of insulin and total cholesterol were determined using enzyme-linked immunosorbent assay.

Results: The composition of gut microbiota in CUG-SGA rats differed from that of NCUG-SGA rats, with reduced abundance of *Lactobacillus* in the CUG-SGA group. The decrease in *Lactobacillus* was significantly associated with increased body weight and upregulated insulin and total cholesterol levels. Five SCFAs and two branched chain fatty acids were significantly higher in the CUG-SGA group than in the NCUG-SGA group. Additionally, SCFAs were positively associated with clinical indices such as weight, body mass index, insulin, and total cholesterol. Transcriptomic data revealed that insulin-like growth factor-2 expression was significantly decreased in CUG-SGA rats and was associated with a decrease in *Lactobacillus* bacteria.

Conclusion: *Lactobacillus* and SCFAs were associated with the metabolic disorders during CUG in SGA. Gut microbiome may play a certain role on metabolic disorders during catch-up growth in small-for-gestational-age.

Keywords: catch-up growth, gut microbiome, *Lactobacillus*, short-chain fatty acids, small for gestational age

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INTRODUCTION

Small for gestational age (SGA) refers to newborns whose birth weight and/or length are at least 2 standard deviations (SDs) below the mean for the gestational age (≤−2 SD) (1). SGA is a common medical problem with a worldwide incidence of 2.3%–10% (2). Newborns with SGA often require additional medical care, including temperature-controlled incubators, tube feeding, and monitoring of blood glucose levels. With advances in medical care, the mortality rate of newborns with SGA has significantly decreased. However, survivors are at increased risks of developing metabolic syndrome and cardiovascular diseases in adulthood compared with infants with normal birth weight (3, 4).

About 85%–90% of infants with SGA demonstrate catch-up growth (CUG) during the first two years of life (5). Worldwide epidemiological data have revealed that CUG of SGA infants may contribute to insulin resistance (6). Eriksson et al found that some SGA infants caught up rapidly after birth, reaching or exceeded the normal body mass index level of children of the same age by 7 years, but with a high death rate associated with cardiovascular disease (7). A cohort study in India found similar results. They found that insulin resistance indicators, total lipoproteins, and low-density lipoproteins were higher in such group of infants (8). However, the relevant mechanism remains unclear.

In the last two decades, our understanding of the causes of SGA has significantly improved, although it still remains limited. For example, fetal genetic deficiencies in the growth hormone/insulin-like growth factor-1 axis have been discovered in SGA (9, 10). Several single nucleotide polymorphisms have been associated with diabetes or obesity in patients born with SGA (9, 11) and maternal placental gene mutations can also cause SGA (12). In addition to genetic factors, other factors such as environmental factors can cause SGA, although the mechanism remains elusive. Whether the intestinal microbiota can impact the host's metabolic activity and contribute to SGA also remains unclear.

In recent years, gut microbiota has been found to be an important environmental factor in the regulation of host metabolism and to significantly impact body weight. Studies have found that the fat content of conventionally raised mice is 40% higher than that of Germ free (GF) mice, independent of food intake (13). When the intestinal microbiota of normal mice is transplanted into GF mice, it changes the body fat composition of the latter (13, 14), suggesting that the intestinal microbiota is involved in regulating the host's ability to obtain energy from the diet. Another study found that transplanting the intestinal microbiota of lean donors into patients with metabolic syndrome improved their insulin sensitivity, implying that the intestinal microbiota is involved in the control of body weight and insulin resistance (15). The intestinal microbiota is involved in the metabolism and transformation of a variety of nutrients, including carbohydrates, proteins, and fats. Studies have found that the intestinal microbiota transforms the indigestible carbohydrates into monosaccharides and short-chain fatty acids (SCFAs). Meanwhile, SCFAs can be used for de novo synthesis of lipids and glucose, thereby affecting the host's glucose and lipid metabolism (16). A cross-sectional study found that the fecal SCFA concentration of overweight or obese subjects is significantly higher than that of thinner controls, and higher SCFA levels have a good correlation with metabolic syndrome indicators (17–20). Studies on genetically obese mice have shown that the intestinal microbiota increases energy utilization by producing excessive SCFAs, thereby affecting the host's metabolism (14). Therefore, we hypothesized that the intestinal microbiota and its metabolites play an important role in host metabolism during the CUG stage in SGA.

We established an SGA rat model and divided these rats into two groups based on their body weight and length —catch-up growth (CUG -SGA) and without catch-up growth (non-catch-up growth, NCUG -SGA). Further, we compared the composition of gut microbiota in these groups. Since the liver is the major metabolic organ, to further verify our hypothesis, we sequenced the transcriptome of liver tissues to identify metabolism-related genes in CUG- and NCUG-SGA rats. The findings of our study will further our understanding of SGA in humans.

MATERIALS AND METHODS

Animal Model

A rat model of SGA was established in animals under maternal undernutrition during pregnancy as previously described (21, 22). In brief, adult SPF-grade Sprague-Dawley female (n=15) and male (n=5) rats were mated overnight. The pregnant rats were randomly divided into SGA group (n=10) and normal control group (n=5). Pregnant rats in the normal control group had standard food diet available *ad libitum* throughout pregnancy, while pregnant rats in the SGA group were under food restriction, with 50% reduction of food intake (about 10 g) from the first day after conception. Rats in both groups gave birth naturally. All newborns were breastfed for the first 3 weeks after birth, and then fed with standard chow. The litter size was culled to five pups per litter at birth in the SGA group and the normal control group, to ensure the catch-up growth of the offspring with SGA. Newborns were defined as SGA rats when the birth weight and length were -2SD below those of the newborns in the normal control. Body weight and length were monitored weekly. NCUG-SGA rats were identified when the body weight and body length were -2SD lower than those in the normal group at week 4 following parturition, and the rest were labelled as CUG-SGA rats. All procedures in this study were approved by the animal experiment ethics committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

Enzyme-Linked Immunosorbent Assay (ELISA)

Commercial ELISA kits were used to measure fasting insulin (Crystal Chem, Illinois, USA), IGF-1 (Crystal Chem, Illinois, USA), and total cholesterol (Zhuocai Biological, Shanghai, China), following the manufacturers' protocols.

Fecal Genomic DNA Extraction and 16S rRNA High-Throughput Sequencing

Five 4-week-old pups were randomly selected from each of the two groups (10 in total, all from separate cages), and their feces collected for 16S rRNA sequencing and SCFA quantification.

Fecal genomic DNA was extracted using TIA Namp Stool DNA Kit (Invitrogen, California, USA) according to the manufacturer's protocol. DNA concentration and integrity were determined using gel electrophoresis. PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 520F (5'- AYTGGGYDTAAAGNG -3') and the reverse primer 802R (5'-TACNVGGGTATCTAATCC -3'). Thermal cycling consisted of initial denaturation at 98°C for 5 min, followed by 25 cycles consisting of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, with a final extension of 5 min at 72°C. PCR products were purified with AmpureXp beads (AGENCOURT), diluted, denatured into single-strands in NaOH buffer, and sequenced on an Illumina Hiseq2500 platform. The raw sequence reads were then processed based on sequence quality. After quality control, the raw reads were classified based on index and barcode, and clustered into operational taxonomic units using QIIME2 dada2 or Vsearch software (https://docs.qiime2.org/2019.7/citation/). Sequencing data analyses were mainly performed using QIIME2 and R packages (v3.2.0). ASV-level alpha diversity indices, such as Chao1 richness estimator, were calculated using the ASV table in QIIME2, by Kruskal-Wallis test, and visualized as box plots. Wilcoxon rank-sum test was utilized to detect differentially enriched microbiota at genus between NCUG-SGA and CUG-SGA groups ($P < 0.05$). Beta diversity analysis was performed to investigate the structural variation of microbial communities among different samples using Bray-Curtis metrics and visualized *via* principal coordinate analysis (PCoA). Non-parametric permutational multivariate analysis of variance (PERMANOVA) was conducted for analyzing significant difference of microbiota structure between the two groups. LEfSe (Linear discriminant analysis effect size) was performed to detect differentially abundant taxa in both groups (23). Microbial functions were predicted by PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) upon KEGG (https://www.kegg.jp/) databases (24).

Fecal SCFA Detection

Feces from the 4-week-old rats were thawed on ice, and approximately 10 mg of feces were homogenized in 50 µl of 15% phosphoric acid. The suspensions were homogenized with a vortex and centrifuged at 12,000 rmp for 10min at 4°C. The supernatants were processed for gas chromatography-mass spectrometry on an Agilent 7890A/5975C mass spectrometer (Agilent Technologies, California, USA). The injection volume was 1 µl, and the split ratio was 10:1. Samples were separated with an Agilent HP-INNOWAX capillary GC column (30 m × 0.25 mm ID × 0.25 µm). The initial temperature was 90°C and was increased to 120°C at 10°C/min, after which the temperature was increased to 150°C at 5°C /min and then to 250°C at 25°C /min, where it remained for 2 min. The carrier gas was helium (1.0 ml/min). The temperatures of the injection port and ion source were 250°C and 230°C respectively under SIM model. Agilent MSD ChemStation software was used for quantitative analysis of chromatographic peak area and retention time (25).

RNA Extraction, Library Construction, Transcriptome Sequencing and Bioinformatics Analysis

Rat liver tissues were stored in liquid nitrogen prior to homogenization with a pestle and total RNA was extracted using Trizol reagent (Invitrogen, California, USA) according to the manufacturer's instructions. RNA integrity was analyzed *via* gel electrophoresis using 1% agarose gel. RNA concentration and quality were measured using a Nanodrop 2000 spectrophotometer (Thermo, Massachusetts, USA). RNA samples (3 µg) were used for library construction using a TruSeq RNA kit (Illumina, California, USA), following the manufacturer's protocol. Clone library was quality-checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) and processed for Paired-end sequencing using Next-Generation Sequencing technology on the Illumina MiSeq platform (Illumina, California, USA).

After sequencing, the images were first converted into raw data in FASTQ format and filtered using Cutadapt (v1.15) software to obtain high-quality reads (clean reads) and analyzed for Q20, Q30 and GC. The filtered clean reads were then mapped to the reference genome using the HISAT2 software (http://ccb.jhu.edu/software/hisat2/index.shtml) to obtain CDS (coding region), Intron (intron), Intergenic (intergenic region) and UTR (5' and 3' untranslated regions) sequences. Gene expression was calculated using the HTSeq Read Count and those with a normalized FPKM value >1 were considered to be expressed. DESeq analysis of RNA-Seq data was set as log2 fold change > 1 or <-1 and P value < 0.05. The volcano map of DEG was generated using the "ggplots2" package in R. DAVID software (6.8) was used for GO and KEGG pathway analysis of the differentially expressed genes. A protein-protein interaction (PPI) network was constructed *via* STRING (Search3 Tool for the Retrieval of Interacting Genes/Proteins), and visualized using Cytoscape software.

RNA-Seq Validation by qRT-PCR

To verify the accuracy of RNA-Seq, Igf2, Mmp14, and Hgf were selected from among the differentially expressed genes, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. After being transcribed into cDNA, PCR was performed on a TIB8600 system (Taipu Biosciences, China) using a 2×SYBR real-time PCR premixture reagent. The primers were designed using Primer Premier 5.0 software and synthesized by Personalbio, Shanghai, China. The relative expression of each gene was calculated using the $2^{-\triangle\triangle Ct}$ method and data were expressed as the mean ± SD. The specific primers used are shown in S1.

Statistical Analysis

GraphPad Prism 8.0 (GraphPad Software, California, USA) and SPSS 23.0 (IBM, Chicago, USA) were used for analyzing data and drawing charts. Normally distributed data was expressed as the mean ± standard deviation (X ± SD). Pairwise differences between groups were analyzed using *t* test. Correlation analysis was performed using Spearman correlation analysis. Correlation coefficient (r) values > 0.6 were considered to represent strong correlations. P values lower than 0.05 were considered significant: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

RESULTS

Characteristics of Rats With NCUG-SGA and CUG-SGA

We first characterized the SGA model in our study. Normal pups are born with normal body weight and length . As described in the methods above, SGA rats are defined as body weight and body length -2SD below the rats from control group at birth, i.e. rats appropriate for gestational age at birth. There were 70 offspring of pregnant rats in the SGA group with restricted diet, including 8 normal weight pups and 62 SGA pups. To ensure catch-up growth of the SGA pups, the litter size was culled to five pups per litter at birth, a total of 50 SGA rats were left. All newborns were breastfed for the first 3 weeks after birth, and then fed standard food. Although given the same food, 26 SGA rats exhibited CUG while other 24 did not. NCUG-SGA rats were identified by week 4, when their body weights were -2SD lower than those in the normal group, and the rest of the SGA rats were defined as CUG-SGA. There were significant differences in the body weight, length , and BMI between rats with CUG-SGA and NCUG-SGA (**Figures 1A–C**). Thus, 4-week-old rats were used in further investigations.

Since CUG in SGA has been associated with increased insulin resistance and metabolic syndrome in humans, we measured if this is consistent with our rat model. Serological analysis showed that both fasting insulin (INS) and total cholesterol concentrations in CUG-SGA rats were higher than those in the NCUG-SGA rats (**Figures 1D, E**).

Differences in the Gut Microbiota Between Rats With NCUG-SGA and CUG-SGA as Revealed by 16S rRNA Sequencing

To compare the microbiota profiles between the NCUG-SGA and CUG-SGA rats, we performed 16S rRNA high-throughput sequencing on rat feces. We first compared the microbiota diversity of individual samples, as indicated by the α diversity index (**Figure 2A**). In both groups, we observed relatively diverse microbiota and variations within each group, without significant difference between NCUG-SGA and CUG-SGA. When we compared the microbiota between the two groups, as indicated by the β diversity index, gut microbiota clearly clustered separately in the NCUG-SGA and CUG-SGA groups, indicating that despite individual variations within the same group, the composition of the gut microbiota differed in these two groups (**Figure 2B**). We further ran the linear discriminant analysis (LDA) effect size algorithm (LEfSe) analysis to evaluate the classification difference of the microbiota between the NCUG-SGA and CUG-SGA groups (**Figures 2D–F**).

It is possible that the difference in microbiota could be the driving force for the differences observed in host growth and metabolism. Therefore, we used the relative abundance results to identify the genera of gut microbiota that are associated with

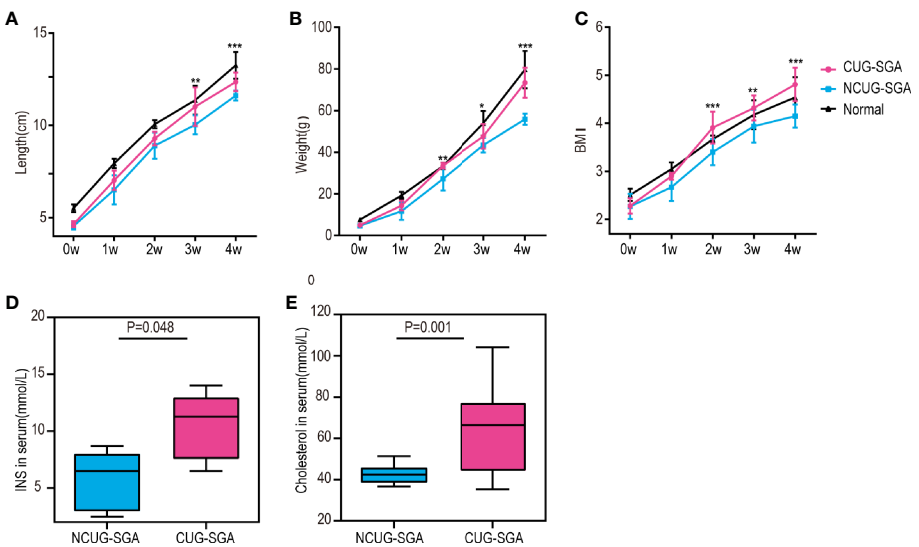

FIGURE 1 | General characteristics of rats between catch-up growth in small for gestational age (CUG-SGA) and non-catch-up growth in small for gestational age (NCUG-SGA) groups **(A)** Body length, **(B)** body weight, and **(C)** Body mass index (BMI) were tracked weekly. Insulin (INS) **(D)** and total cholesterol **(E)** were measured at week 4. CUG-SGA (n=13) and NCUG-SGA(n=12). *p< 0.5, **p < 0.01, ***p < 0.001 (NCUG-SGA vs CUG-SGA).

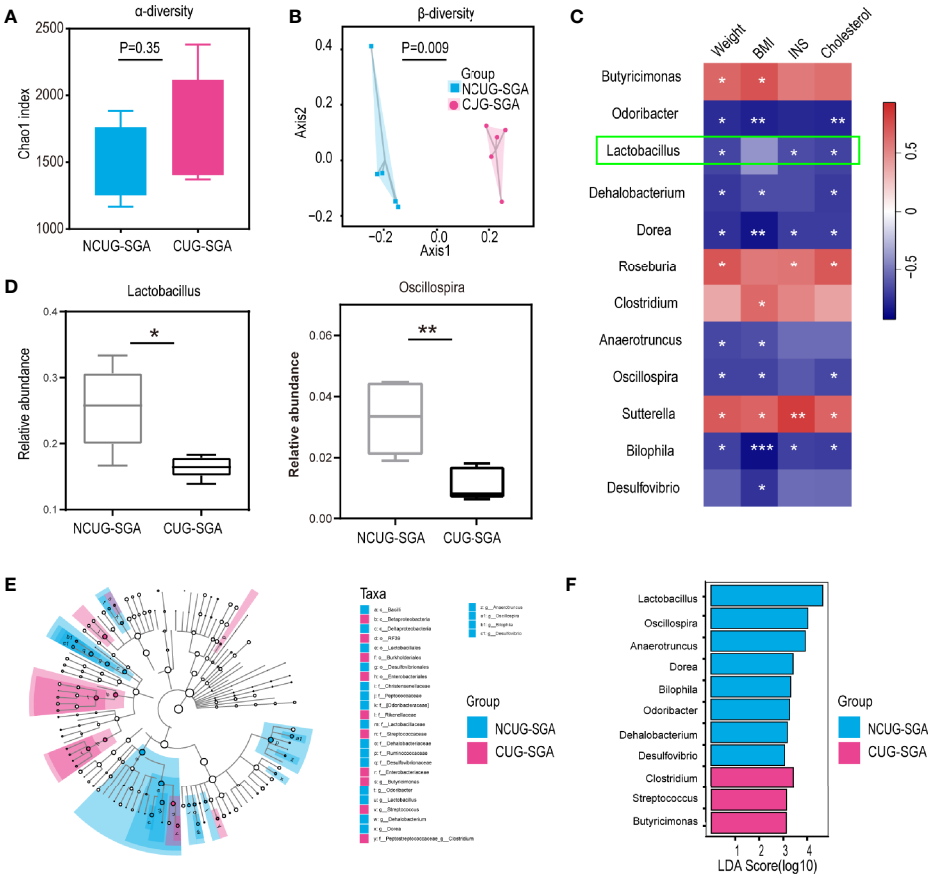

FIGURE 2 | Variations in intestinal microbiota between non-catch-up growth in small for gestational age (NCUG-SGA) and catch-up growth in small for gestational age (CUG-SGA) rats **(A)** The α diversity of the intestinal microbiota of rats between the two groups (Chao1 index) showed that both groups have variations among individual samples within each group, but not significant difference between the two groups; **(B)** Comparison of the β diversity of the intestinal microbiota based on the Bray-Curtis distance shows significant difference between the NCUG- and CUG-SGA rats; **(C)** A heatmap of Spearman correlation between microbiota at genus level and clinical characteristics; **(D)** Box plots showing the abundance of Lactobacillus and Bilophila in the NCUG-SGA and CUG-SGA groups; **(E)** Differences of classification unit between the groups; **(F)** Linear discriminant analysis (LDA) effect size algorithm (LEfSe) of intestinal microbiota (LDA>3).

body weight, BMI, INS, and total cholesterol. As shown in **Figure 2C**, *Butyricimonas, Roseburia, Clostridium*, and *Sutterella* were positively correlated with body weight, BMI, INS level, and/or total cholesterol level respectively, while *Odoribacter*, *Lactobacillus*, *Dehalobacterium*, *Dorea, Anaerotruncus, Oscillospira, Bilophila*, and *Desulfovibrio* were negatively associated with body weight, BMI, INS and/or cholesterol level, suggesting that among the thousands of bacteria detected, these 12 different genera may play an important role in shaping the host body weight and metabolic activity.

Fecal SCFA Content Is Higher in the CUG-SGA Group Than in the NCUG-SGA Group

Gut microbiota and their metabolic products play a critical role in shaping host metabolism and growth, as shown by the defects observed in GF animals (13, 14). However, the16s rRNA sequencing data does not provide functional profile directly. To further understand the potential effect of the microbiota on host in SGA, we used PICRUSt2 for functional prediction. We found that the abundance of 30 KEGG pathways was significantly different between the NCUG-SGA and CUG-SGA groups. The pathways enriched in the CUG-SGA group include the pathways of fatty acid biosynthesis, propionate metabolism, and butanoate metabolism, which implied that SCFAs may play an important role in the mechanism of CUG and its metabolic change (**Figure 3A**).

SCFAs, one of the main metabolites of the gut microbiota, play a role in energy regulation and metabolism by acting as intermediaries between the gut microbiota and the host. To examine the potential role of SCFAs in regulating host metabolism during CUG, we first measured SCFA concentrations in the feces of the rats at four weeks of age. As shown in **Figure 3B**, there were five SCFAs (acetic acid, propionic acid, butyric acid, valeric acid and caproic acid) and two branched chain fatty acids (iso butyric acid, isovaleric acid), that were significantly higher in the feces of rats in the CUG-SGA group than in the NCUG-SGA group (all p values < 0.05), which was consistent with the prediction results of intestinal microbiota function.

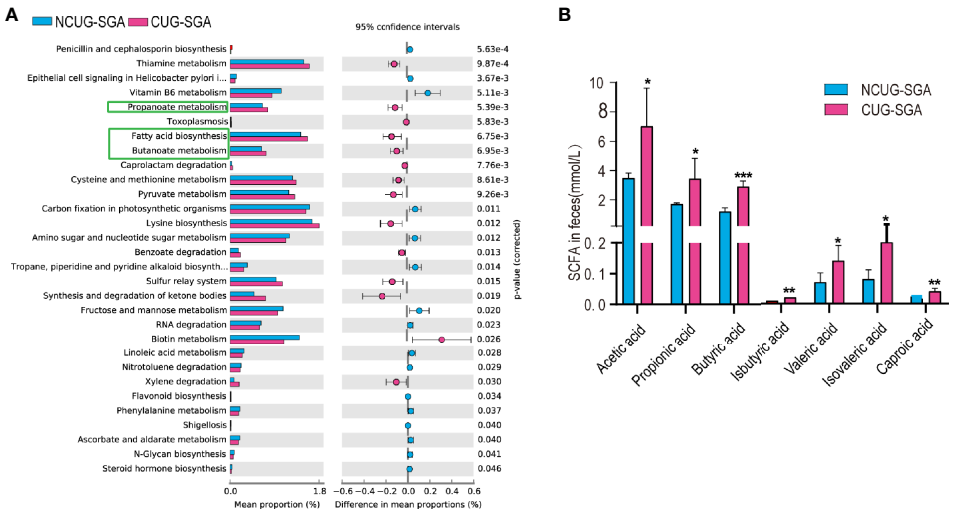

FIGURE 3 | Intestinal microbiota functional prediction and short-chain fatty acid (SCFA) detection. **(A)** KEGG pathway analysis based on 16S rRNA between NCUG-SGA and CUG-SGA groups in rats, among them, fatty acid biosynthesis, propionate metabolism, and butanoate metabolism are significantly upregulated in the CUG-SGA group rats. **(B)** Detection of SCFA from fecal samples in NCUG-SGA and CUG-SGA groups. *p < 0.5, **p < 0.01, ***p < 0.001. CUG-SGA, catch-up growth in small for gestational age; NCUG-SGA, non-catch-up growth in small for gestational age.

SCFAs Are Associated With the Gut Microbiota and Clinal Features in SGA

The function prediction of gut microbiota and SCFA levels suggested that the gut microbiota may play an important role in metabolic syndrome in CUG in SGA through SCFA. First, we established a correlation matrix based on the Spearman rank correlation coefficient to explore the relationship between intestinal microbiota and fecal SCFA levels. Eleven different bacterial genera were significantly related to at least one SCFA ($P < 0.05$, $|r|>0.6$), indicating that SCFA levels were closely related to the intestinal microbiota. Among them, *Lactobacillus*, *Bilophila*, *Oscillospira*, and *Desulfovibrio* had a significant negative correlation with different SCFAs ($P < 0.05$, $r < -0.6$). The concentration of butyric acid was positively correlated with the abundance of butyric acid-producing *Butyricimonas* ($P < 0.05$, $r > 0.6$) (**Figure 4A**).

Spearman correlation analysis was performed to determine the correlation between the clinical indicators of NCUG-SGA and CUG-SGA rats and SCFAs. The results showed that five SCFAs and two branched chain fatty acids had a significant positive correlation with at least one clinical index (weight, BMI, insulin, and total cholesterol), indicating that SCFAs may affect the metabolism of the host (**Figure 4B**). These findings suggest that gut microbiota and SCFAs may play a role in the metabolic syndrome associated with the catch-up growth of SGA.

Changes in Metabolism-Related Pathways in the Liver Transcriptome in CUG-SGA Rats With Decreased Igf2 Expression

The study on the gut microbiota of SGA rats indicated that SCFAs may be involved in the metabolic disorders in the process of catch-up growth. The liver is an important metabolic organ in mammals, and it establishes connections with other tissues of the body through its metabolic function. To clarify the correlation between SCFAs and the metabolic disorders associated with CUG in SGA, we sequenced the transcriptome of liver tissues in NCUG-SGA and CUG-SGA rats. Results revealed 2416 differentially expressed genes (DEGs), of which 1403 were upregulated and 1013 were downregulated (**Figure 5A**). To clarify the biochemical metabolism and signal transduction pathways involved in DEGs, we conducted KEGG pathway analysis and found significant differences in 53 metabolic pathways ($P<0.05$). **Figure 5B** shows the top 34 metabolic pathways, including protein synthesis, fatty acid metabolism, AMPK signaling, insulin signaling, PPAR signaling, MAPK signaling, and insulin resistance. These results provide important clues for studying the metabolic syndrome in CUG in SGA.

To systematically analyze the functions of DEGs in the liver tissues of the NCUG-SGA and CUG-SGA groups, we mapped the DEGs to the protein-protein interaction (PPI) (https://string-db.org/) database and generated the PPI network diagram **(S2)**. Igf2, Mmp14, and Hgf were significantly different between the two groups, and showed interactions with each other (**Figure 5C**), Igf2 was very close to the gene encoding insulin on human chromosome 11p, which may contribute to regulate the body weight and obesity in children and adults (26); Mmp14 and Hgf were also associated with insulin resistance (27, 28), and these three genes are members of MAPK pathway mentioned above. Igf-2 was significantly downregulated in the CUG-SGA group. The expression of Hgf and Mmp14 significantly increased in the CUG-SGA group. These results were verified using qRT-PCR, indicating that transcriptome sequencing can reliably reflect the changes in gene expression (**Figure 5D**).

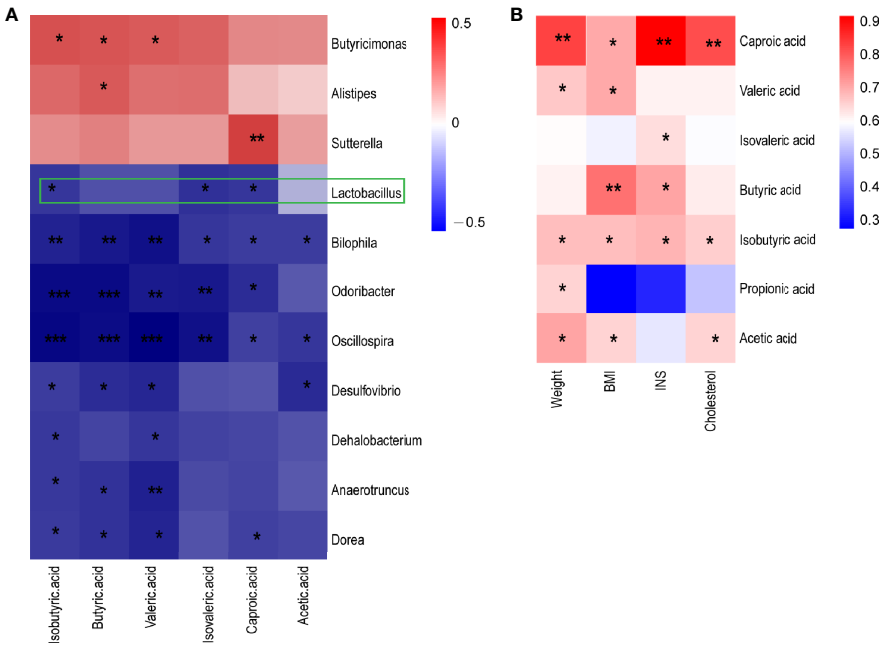

FIGURE 4 | A heatmap of Spearman correlation between intestinal microbiota at genus level and metabolism index and SCFA **(A)** A heatmap of Spearman correlation between microbiota at genus level and SCFAs. **(B)** A heatmap of Spearman correlation between clinical characteristics and SCFAs. Red: positive correlation; blue: negative correlation; *P < 0.05, **P < 0.01, ***P < 0.001. SCFAs, short-chain fatty acids.

Correlation analysis showed that Igf2, Hgf, and Mmp14 were significantly correlated with clinical metabolic indicators (weight, BMI, INS, and TC) (P<0.05, |r|>0.6) (**Figure 5E**), indicating that these three genes may be involved in metabolic changes during SGA growth catch-up. To clarify the correlation between DEGs and intestinal microbiota , we analyzed the correlation between Igf2, Hgf, and Mmp14 and the gut microbiota and SCFA. The results showed that Igf2 and Hgf were significantly related to the abundance of *Lactobacillus* (P<0.05, |r|>0.7) (**Figure 5F**); the expression levels of Igf2, Hgf, and Mmp14 were related to the changes in SCFA (P<0.05, |r| >0.6) (**Figure 5G**).

DISCUSSION

Insulin resistance and metabolic syndrome greatly affect human life expectancy and quality of life. A large number of studies have found that the risk of insulin resistance and obesity is significantly increased in the growth of SGA individuals, and the metabolic disorders are related to CUG. In this study, we aimed to evaluate the role of gut microbiota independent of genetic factors in the process of CUG in SGA. Pregnant rats were given restricted food (reduced by 50% from the first day of gestation until delivery as compared to normal diet) to give birth to SGA pups. At 4 weeks, the length and weight of CUG-SGA rats were significantly higher than those in the NCUG-SGA group, with elevated levels of insulin and total cholesterol. This was associated with significant differences in the diversity and composition of gut microbiota between these two groups, including *Lactobacillus*, whose relative abundance was most significantly decreased in the CUG-SGA group. SCFA levels in the CUG-SGA group were significantly increased, with significant changes in the mRNA level of IGF-2 as shown by the liver transcriptome analysis. Our findings suggest that the gut microbiota and its metabolites may relate to the metabolic process in the liver and may be associated with increased body weight and the systemic metabolic dysfunction in the host.

To study the relationship between gut microbiota and host metabolism, we used different techniques to capture and present a complete and unbiased picture, including 16s rRNA sequencing, SCFA detection, and liver tissue RNA sequencing. This allowed us to identify the decrease in *Lactobacillus* as the most significant event across different analyses in CUG-SGA as compared to the NCUG-SGA group. The gram-positive *Lactobacillus* is the dominant bacteria in the gastrointestinal microbiota of mammals and can affect the composition and metabolism of gut microbiota by producing metabolites such as lactic acid which lowers pH (29, 30). The impact of *Lactobacillus* on weight change varied according to the species: some species were associated with weight gain, while others were linked to weight loss. Meta-analyses have shown that *Lactobacillus* can promote loss of weight and fat in overweight adults and children (31, 32). *Lactobacillus* strains isolated from the feces of super-longevity people reduced serum cholesterol levels in rats with a high-cholesterol diet (33). *Lactobacillus* selected from fermented cow milk also reduced serum cholesterol levels in rats (34). Moreover, the effect of the same strain of Lactobacillus could be

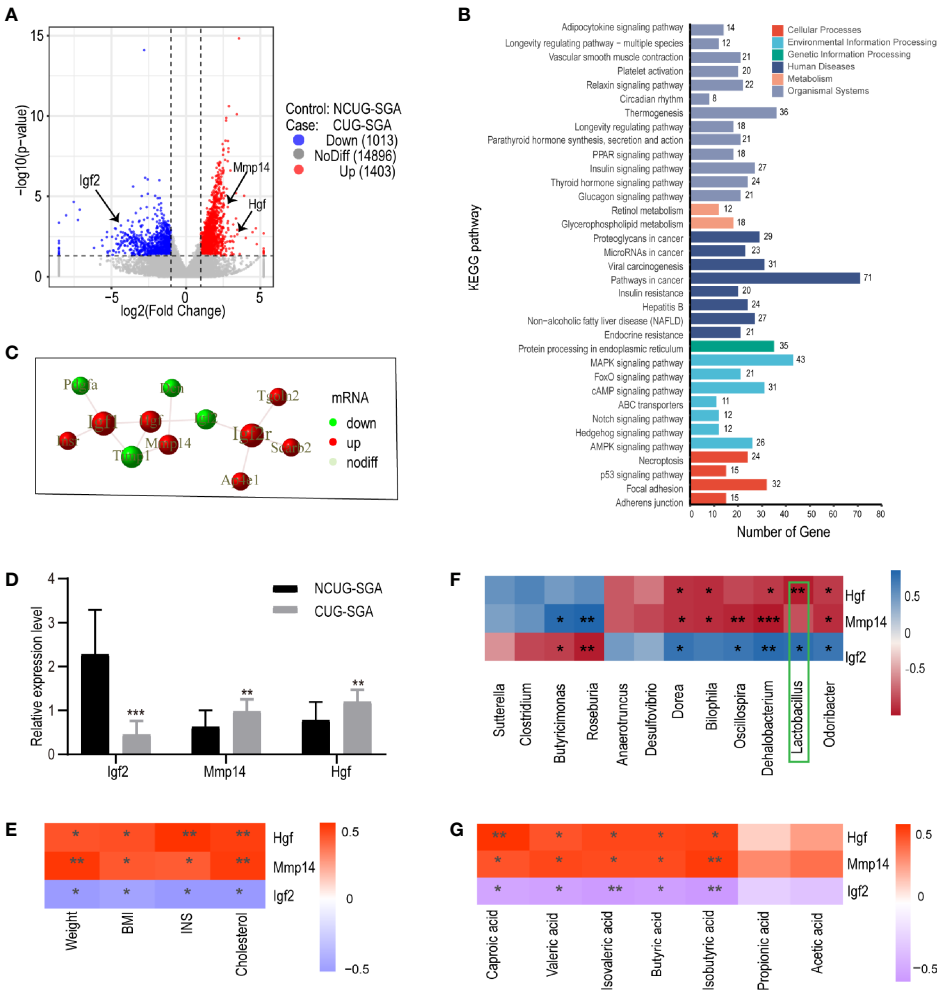

FIGURE 5 | Transcriptome sequencing, validation, and correlation analysis of metabolism-related genes with microbiota and clinical indicators in liver tissues of rats from NCUG-SGA (n=5) and CUG-SGA (n=5) groups **(A)** Volcano plots of differentially expressed genes (DEGs) with |log2FoldChange|>1 and P value<0.05; **(B)** KEGG pathways of DEGs; **(C)** the protein-protein interaction (PPI) of Igf2, Mmp14, and Hgf; **(D)** qRT-PCR validation of metabolism-related genes; **(E)** Correlation analysis between metabolism-related genes and clinical indicators; **(F)** Correlation analysis between metabolism-related genes and microbiota at genus level; **(G)** Correlation analysis between metabolism-related genes and SCFA. *P < 0.05, **P <0.01, ***P< 0.001.

different depending on the species of the host (35). In our current study, the increase in body weight, insulin, and total cholesterol in the CUG group was significantly associated with decreased abundance of *Lactobacillus*. Therefore, the metabolic disorder of catch-up of SGA may be related to the decrease in *Lactobacillus* and other bacteria.

Furthermore, our study explored the changes of metabolites such as SCFAs. PICRUSt2s functional prediction based on 16S rRNA sequencing data showed that CUG-SGA rats microbiome were associated with metabolic pathways including fatty acid, propionate, butyrate, etc. In consistency, the contents of fecal SCFAs were significantly higher in the CUG-SGA group than in the NCUG-SGA group. Correlation analysis showed that the increase in SCFAs was related to the decrease in *Lactobacillus*, *Bilophila*, *Odoribacter*, and *Oscillospira*. As the main metabolite of the intestinal microbiota, SCFAs act as intermediaries between the gut microbiota and the host, and their composition is closely related to the intestinal microbiota. Numerous studies have demonstrated that SCFAs play a positive role in the energy metabolism of mammals. After absorption, SCFAs could be used as energy sources for the host and for regulating energy metabolism (36). In the human body, SCFAs provide about 10% of the daily caloric requirement (37). SCFAs are transported to the liver through the portal vein where butyric acid could stimulate the expression of genes associated with gluconeogenesis (38).Butyric acid has also been shown to be metabolized by acetyl CoA for the synthesis of fatty acids, cholesterol and ketone body through acetyl-CoA metabolism, thereby providing specific substrates for lipid biosynthesis (39). Butyric acid and propionic acid also act as ligands for G protein-coupled receptors Gpr41 and Gpr43 and affect the body's metabolism (40–42). Moreover, changes in

SCFA levels may contribute to metabolic disorders such as insulin resistance and obesity. In our study, the increase in SCFA levels may be associated with the metabolic disorders during CUG in SGA. It has to be mentioned that the fecal SCFAs are only a fraction of the total SCFAs and thus changes of fecal SCFAs may not accurately reflect the changes in total SCFAs (37). In addition, it has been proposed that increased SCFAs contributes to the development of obesity, this remains controversial (43).

The metabolism of SCFAs depend on the enterohepatic circulation. By comparing the transcriptome sequences of rat livers in the NCUG-SGA and CUG-SGA groups, we found that the DEGs were mainly involved in insulin response, and carbohydrate, protein, and fatty acid metabolism. Among them, Igf2 was significantly reduced in the CUG group. Studies have found that lower Igf2 concentration is associated with weight gain and obesity (44). Murphy et al. have reported a case of a patient harboring an Igf2 defect combined with insulin resistance and lipid metabolism disorders (45). The Igf2 imprinting gene is very close to the gene encoding insulin on human chromosome 11p, which may play an important role in regulating the body weight and fat in children and adults (26, 46–50). *In vivo* assay showed that decreased Igf2 expression occurred with fat deposition and obesity, which may not be caused by an increase in food intake, but more likely by changes in energy homeostasis (51).

PPI networks suggested that Igf2 may interact with Hgf and Mmp14. Hgf stimulates glucose uptake and metabolism in mouse skeletal muscles and plays an important role in insulin resistance (27). In humans, serum Hgf is significantly higher in patients with obesity, metabolic syndrome, and diabetes compared to healthy subjects (52–54). A prospective study revealed that elevation of serum Hgf is significantly correlated with insulin resistance (27). Mmp14, Igf2, and Hgf are members of MAPK pathway. The circulating glucose and triglyceride levels in Mmp14-knockout mice decreased by 50% compared to levels in wild type mice, as demonstrated by reduced tissue glycogen and lipid levels, and plasma glucose and triglycerides (28). Therefore, we speculated that Igf2, Hgf, and Mmp14 may be involved in the metabolic changes in CUG in SGA. Correlation analysis showed that the expression levels of Igf2, Hgf and Mmp14 were associated with the abundance of *Lactobacillus* and SCFA contents. It is possible that the metabolites of *Lactobacillus* and other SCFAs are detected by the host, leading to altered expressions of Igf2, Hgf and Mmp14, which then regulate the host blood glucose and triglyceride levels and insulin sensitivity in SGA. The detailed mechanism needs further research.

In the present study, we found that *Lactobacillus* may affect the host's body weight and insulin and cholesterol levels by regulating SCFAs during the catch-up process in SGA rats. Changes in *Lactobacillus* levels were associated with increased levels of SCFAs. Following transportation into the liver *via* the enterohepatic circulation, SCFA may be related to the decreasing Igf2, associated with insulin resistance and lipid metabolism disorder. Although the relationships between the microbiota, SCFAs and liver transcriptomes are based on computational analysis and predictions, the consistent findings combining three different profiles of the SGA rats strengthened our study. These findings provide a theoretical basis for elucidating the metabolism and developing novel therapies in SGA patients, especially those with CUG.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI PRJNA679705.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experimental Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

AUTHOR CONTRIBUTIONS

ZD and XW proposed the initial experimental ideas. JA, ZD, YX, WL, and XW developed the experimental design. JA, JW, LL, and LC conducted the experiments. JA, ZD, XW, and LG wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Impaired Intestinal Barrier and Tissue Bacteria: Pathomechanisms for Metabolic Diseases

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An intact intestinal barrier, representing the interface between inner and outer environments, is an integral regulator of health. Among several factors, bacteria and their products have been evidenced to contribute to gut barrier impairment and its increased permeability. Alterations of tight junction integrity - caused by both external factors and host metabolic state - are important for gut barrier, since they can lead to increased influx of bacteria or bacterial components (endotoxin, bacterial DNA, metabolites) into the host circulation. Increased systemic levels of bacterial endotoxins and DNA have been associated with an impaired metabolic host status, manifested in obesity, insulin resistance, and associated cardiovascular complications. Bacterial components and cells are distributed to peripheral tissues *via* the blood stream, possibly contributing to metabolic diseases by increasing chronic pro-inflammatory signals at both tissue and systemic levels. This response is, along with other yet unknown mechanisms, mediated by toll like receptor (TLR) transduction and increased expression of pro-inflammatory cytokines, which in turn can further increase intestinal permeability leading to a detrimental positive feedback loop. The modulation of gut barrier function through nutritional and other interventions, including manipulation of gut microbiota, may represent a potential prevention and treatment target for metabolic diseases.

Keywords: obesity, metabolic disease, intestinal permeability, microbiome, zonulin, adipose tissue microbiota, type 2 diabetes, endotoxemia

INTRODUCTION

While the obesity and type 2 diabetes (T2D) pandemics are increasing at a fast pace (1), new factors relevant to the lack of adaptation toward the increasingly rapid changes in our environment have been proposed as possible perpetrators. Among those, the intestinal microbiota and its interactions with host metabolism and the immune system have been acknowledged to influence and contribute to several diseases including gastrointestinal disorders such as inflammatory bowel diseases (2) and more recently the plethora of metabolic and cardiovascular diseases (3, 4). However, the underlying mechanisms are still unknown (5–7). One poorly understood feature of metabolic disease is the alteration and dysfunction of the intestinal barrier, accompanied with an increase in intestinal permeability. The healthy gut barrier constitutes a crucial boundary protecting the host from external stimuli and pathogens by providing spatial compartmentalization and various defense mechanisms, while concurrently allowing the uptake of necessary nutrients (8). While the influx of microbial products has been suggested to underlie chronic inflammation observed in metabolic disease and more specifically T2D (9), clinical features of metabolic diseases such as hyperglycemia in T2D have been associated with increased influx of microbial products in humans reflective of glucotoxicity (10). First studies in the 1950s suggested that increased endotoxins, which are lipopolysaccharides of gram-negative bacteria in the circulation result from an increased intestinal permeability (11). Although still under debate, independent studies in mice and humans could confirm these results (12–16). In 1984, the term "leaky gut" was first introduced when Bjarnason et al. reported that patients with excessive alcohol consumption had increased intestinal permeability (17), which is also referred to as hyperpermeability (18). In the following years research on intestinal permeability focused on autoimmune and inflammatory diseases such as celiac disease (19, 20) or Crohn's disease (21). Only recently, possible associations between intestinal permeability and characteristics of a metabolic disease like obesity or T2D have emerged as plausible and attractive targets in the field of obesity research.

In this review, we aim to highlight underlying mechanisms of an impaired intestinal barrier and its possible impact on metabolic health. We specifically discuss recent findings on how endotoxemia and translocation of bacteria, bacterial genetic material and products may cause tissue and organ dysfunction subsequently contributing to metabolic diseases.

THE INTESTINAL BARRIER

Components of Intestinal Barrier

The intestine represents an active interface, where external environmental factors, such as diet, medication and the billions of symbionts inhabiting our gut co-exist and interact – usually peacefully – with host factors including the immune system (**Figure 1A**). The intestinal barrier allows and facilitates the uptake of nutrients and water from the intestinal lumen, whilst at the same time providing effective mechanisms to combat harmful substances and pathogens and prevent their translocation into the host circulation and peripheral tissues (22) (**Figure 1B**). These checkpoints comprise an intricate network of mechanical and immunological factors including the mucus, the epithelium and the underlying lamina propria (22) as well as humoral and immunological factors. The goblet cells produced mucus layer constitutes of mucin and forms a protective barrier, limiting the amount of bacteria reaching the epithelial cells (23). Epithelial cells form microvilli, protrusions in the range of 100 nm width, that help to increase the area for absorptions of ions and nutrients using specified receptors (24). This line of densely arranged microvilli is termed brush border, which, on the one hand, physically prevents bacteria coming in contact with the cell soma, but otherwise help to actively combat pathogenic bacteria (25). For example, native luminal vesicles containing intestinal alkaline phosphatase are released from the tips of the microvilli, helping to dephosphorylate lipopolysacchradies (LPS), hinder bacterial growth and prevent adherence of enteropathogenic *E. coli* to the epithelial cell layer (26). Epithelial cells are connected by tight junctions (TJ), which hinder the entry of pathogens while regulating the paracellular flow of water, small ions and nutrients (27). Tight junction complexes mainly consist of occludin and members of the claudin family. Expression of the latter is tissue-specific and the claudin composition defines the kind and function of a specific tight junction, e.g., claudin-7 reduces permeability to anions and claudin-1 reduces permeability to cations (28, 29). Claudins and occludin are connected to the cytoskeleton by scaffolding proteins including zonula occludens (ZO) proteins (30) (**Figure 1C**). Additionally, cells are connected by adherence junctions (for cell-cell signaling) (31) and desmosomes (for cell stability) (32). The mechanical gut barrier interacts constantly with and is highly impacted by local immune components consisting of soluble Immunoglobulin A (IgA), epithelial and Paneth cell produced, and secreted antimicrobial peptides such as alpha-defensins, lysozyme, and C-type lectin (33) providing additional protection to the epithelial layer and the crypts of Lieberkühn (34). Not less important are cells belonging to the innate and adaptive immunity system such as macrophages, dendritic cells (35), T-regulatory cells (36) and the highly dense lymphoid tissues inhabiting the lining of the gut but also highly active in adjunct lymph nodes (37). Disruptions in this intricately balanced system through toxins, microorganisms, nutrients, or food contaminants can lead to an alteration of the intestinal permeability, by an increased and inadequately controlled influx *via* the paracellular and transcellular pathway.

The regulation of this system can, under both physiological and pathophysiological conditions, be altered by various endogenous and exogenous factors as discussed in the following paragraph.

Endogenous Regulation of the Intestinal Permeability

A constant challenge for upholding the integrity of the intestinal barrier is the continuous cell shedding on the tip of the villi, with a turnaround time of about five days (38). Tight junctions are

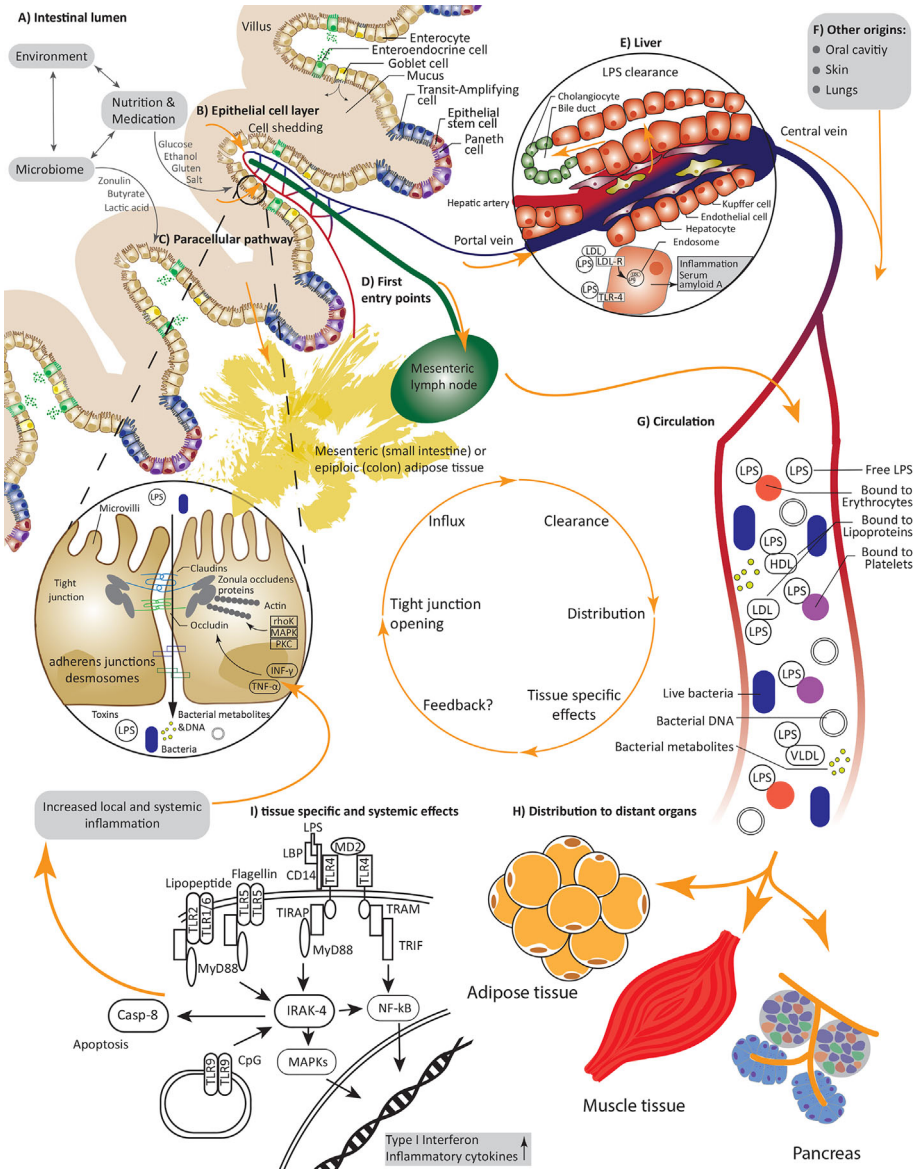

FIGURE 1 | Causes and consequences of increased intestinal permeability; **(A)** An interplay between environmental factors, nutrients, drugs and the microbiome defines our intestinal lumen and can directly or indirectly alter permeability of the epithelial cell layer; **(B)** Increased cell shedding under pathophysiological conditions or the reversible interaction with and opening of tight junctions under allow an influx of pathogens including bacteria or their nucleic acids, metabolites, and lipopolysaccharides as well as further substances and toxins; **(C)** Paracellular transport *via* tight junctions is regulated by various kinases and inflammatory cytokines, nutrients and bacteria can interfere with claudin and occludin expression; **(D)** First entry points of invading substances are capillaries and lymph vessels of the villi and tissues in close proximity such as mesenteric adipose tissue; **(E)** Endotoxin is rapidly and constantly cleared in the liver; **(F)** Next to the gut lumen, pathogens could also invade through the oral cavity, the skin and the lung; **(G)** Under constant interactions and clearance with the immune system the LPS and bacteria are transported in the circulation, thereby LPS can be bound to various proteins and cells including erythrocytes and lipoproteins; **(H)** Effected distant organs include adipose tissue, pancreas tissue and possibly muscle tissue; **(I)** Pathogens interact with many receptors, i.e., LPS is recruited to TLR-4 leading to increased expression and secretion of inflammatory cytokines.

redistributed to block the gap under involvement of rho kinase (39). Other signal cascades involved in tight junctions regulation under physiological conditions include protein kinase C's (PKC) and mitogen-activated protein kinase (MAPK) pathways, which regulate protein phosphorylation and expression allowing a selective transport of molecules (40). In disease, increased proinflammatory cytokines such as interferon-γ (INF γ) and tumor necrosis factor α (TNF-α) shift the homeostatic balance (27) by downregulating the expression of claudin-1 (27) and increasing occludin and claudins-1 and -4 internalization,

leading to an increasingly compromised gut permeability, as shown in T84 cells *via* immunofluorescence microscopy (41). Further mechanisms of INF-γ induced permeability in T84 cells involve phosphatiodylinositol-3-kinase/protein kinase B (PI3-K/Akt) activation and a delayed but prolonged NF-kB response (42) (**Figure 1C**). This is of particular interest, as metabolic diseases are usually attended by a chronic low grade inflammation (43), possibly initiating a detrimental positive feedback loop.

Mechanisms leading to whole bacterial translocation through the mucosal barrier remain unclear. Several studies suggest transcytosis *via* M-cells (44), which uptake large antigens and particulate matter such as viruses, bacteria, and protozoa through receptor mediated endocytosis (45). The transcellular pathway can be triggered and upheld by low concentrations of INF-γ (that are insufficient to alter the paracellular pathway), as shown by Clark et al. in Caco-2 cells (46). This uptake is also dependent on extracellular signal-regulated kinase (ERK) 1/2 and ADP-ribosylation factor (ARF)-6 signaling (25, 47).

Exogenous Perturbation

Several external factors, including bacteria, alcohol consumption, nutrition and medication have an impact on the intestinal permeability (34). A high caloric "western" diet, in particular due to high-fat/low-fiber content (48) and food additives (49), is associated with adverse changes in the microbiome (50) and both are independently important factors in the development of metabolic diseases. Whereas pathogenic bacterial species such as enteropathogenic *E. coli* (51–53), *H. pylori* (54), *S. Typhimurium* (55), and *V. cholera* (56) increase intestinal permeability, the totality of the gut microbiota contributes equally to upholding the gut barrier health by shaping tolerogenic mechanisms controlling the responses of resident dendritic cells and macrophages (57). Pathogenic bacteria can use their type III secretion system to alter the host cytoskeleton in their favor to facilitate entry to epithelial cells (58), but also non-invasive parasites like G. lamblia can introduce adverse changes, in this case by causing microvilli shortening and TJ disruption (25, 59). Moreover, beneficial effects were reported for gut bacteria produced short chain fatty acids (60, 61), in particular butyrate (61, 62), which has been shown to act as an energy source for colonocytes (63), regulate hypoxia induced factor 1α (HIF-1α) dependent expression of many pro-barrier genes in intestinal epithelial cells (64), facilitate tight junctions assembly (61) and induce colonic regulatory T-cells, leading to the suppression of inflammatory and allergic responses (62). Taxonomic groups-wise, *A. muciniphila* (65), Bifidobacterium (66), and lactic acid producing bacteria like *L. plantarum* (67) and *L. reuteri* (68) were, among others, also reported to improve intestinal permeability (**Figure 1A**).

Experimental evidence for the effect of food components mostly stems from rodent and cell culture experiments. Glucose, well known for its many adverse effects in metabolic disease (69), is partially transported paracellularly and enhances small intestinal permeability (70), increases Caco-2 cell permeability and leads to altered TJ arrangements (71). Recently, Thaiss et al. demonstrated a key role for hyperglycemia in impairing the barrier function by increasing retrograde uptake of glucose into epithelial cells *via* the glucose transporter (GLUT)-2 transporter, leading to changes in intracellular glucose metabolism and to transcriptional reprogramming. The latter includes, among others, alterations in the expression of genes involved in N-glycan biosynthesis and pentose-glucuronate interconversion, two pathways linked to the maintenance of epithelial barrier function (10). Further food components increasing TJ permeability include salt (72) and various fatty acids (73), all of which are known to contribute to obesity development (74, 75). Another important nutritional factor affecting the intestinal permeability is gliadin, a component of gluten found in wheat. In mouse experiments, Lammers et al. found that gliadin increased permeability by binding to the chemokine receptor 3 (Cxcr3) (76) (**Figure 1A**). Beneficial effects on intestinal permeability were reported for casein (77) and other cheese peptides (78), vitamin D (79) as well as polyphenols (80, 81), which is in line with findings that polyphenol rich diets ameliorate metabolic disease (82). Furthermore, addition of apple-derived pectin to a high fat diet (HFD) counteracted some negative effects of HFD by preventing microbial shifts and improving barrier function due to increased expression of claudin-1 and abating metabolic endotoxemia (83).

Moreover, occludin and claudin expression are subject to circadian control, associated with regulation of intestinal permeability and susceptibility to colitis (84). This is further substantiated by the increase in intestinal permeability after circadian disruption in mice leading to promotion of alcohol induced liver disease and inflammation (85). Similarly, gut bacterial signatures related to diurnal oscillation also enable the risk stratification and prediction of T2D within 5 years of sampling (86) linking circadian control with gut microbiota profiles and intestinal permeability regulation and downstream metabolic sequalae.

INTESTINAL PERMEABILITY IN METABOLIC DISEASE

Animal Studies

An increased intestinal permeability is the key factor for the migration of toxins and bacterial components to the circulation. First evidence for a link between obesity and increased permeability was found in mouse studies. Brun et al. used Ussing chambers – a physiological system to measure the transport of ions, nutrients, and drugs across epithelial tissues including the gut (87, 88) to analyze the barrier integrity in leptin-deficient (*ob/ob*) and hyperleptinemic, but leptin-receptor mutant (*db/db*) mice, a widely used animal models in obesity-induced T2D (89). They observed a significantly increased permeability, which was more prominent in *db/db* mice. This was accompanied by an increase in portal endotoxemia and systemic inflammation parameters (90). Later, changes in the microbiome were found to be an important factor linking

intestinal permeability to inflammation (91, 92). In a key study, Cani et al. could further show that a HFD resulted in increased levels of plasma endotoxin as well as a reduced expression of TJ genes ZO-1 and occludin. This effect was reduced when mice on HFD were treated with antibiotics and plasma endotoxin levels were positively correlated with markers of inflammation (Plasminogen-Aktivator-Inhibitor 1, *PAI-1*) and oxidative stress (six transmembrane protein of prostate 2, *STAMP2*) (9). It was further shown that the shift in the intestinal permeability of mice on HFD could be attributed to claudin switching, which leads to tight junction restructuring. In this case, the expression of claudin-1,-3,-4,-7, and -15 decreased, whereas expression of claudin-2 increased (93). However, as shown in *db/db* diabetic mice with induced *C. rodentium* infection, physiological countermeasures are in place to overcome initial intestinal barrier impairment. In particular, exogenous IL-22 restored mucosal host defense as indicated by histological evaluation (94).

Human Studies

Although some studies have linked intestinal permeability with metabolic disease in humans, they should be seen with caution, as they did not employ reproducible and robust methods to test for gut permeability such as lactulose/mannitol (La/Ma) or similar tests and Ussing chambers. In 2011 Gummesson et al. reported an association between intestinal permeability and increased visceral obesity in 67 otherwise healthy women (95) and in 2012, Teixeira et al. corroborated these finding by showing that increased permeability is associated with increased BMI and insulin resistance in 40 patients with obesity and T2D (96). Damms-Machado et al. similarly reported a correlation between intestinal permeability and Homeostasis Model Assessment for insulin resistance (HOMA-IR) in 27 individuals with obesity of which 18 suffered from the metabolic syndrome (97). Furthermore, Luther et al. reported in a meta-analysis showing that nonalcoholic fatty liver disease (NAFLD) was associated with increased intestinal permeability (odds ratio increased intestinal permeability=5.1), which was even more prominent in nonalcoholic steatohepatitis (NASH) (odds ratio=7.2) (98). Challenging this hypothesis with interventions has seldom been done: A low-caloric diet of 800 kcal/day was able to significantly reduce the intestinal permeability, indicating the relevance of nutrition as a fast acting player influencing intestinal permeability (99). Further interventions leading to mechanistic insights in the link between metabolism and intestinal permeability are highly warranted.

METABOLIC ENDOTOXEMIA AND THE TRANSLOCATION OF BACTERIAL GENETIC MATERIAL AND LIVE BACTERIA IN HUMAN TISSUES: A COMPARTMENT CENTERED APPROACH

The evidence related to translocation of bacterial components, live bacteria, and bacterial metabolites into the circulation and beyond is highly dependent on the tissue studied leading to diverging degrees of confidence in the relationship between gut permeability, organ dysfunction and overall modulation of host metabolism. On their way from the gut, bacteria are enriched in mesenteric lymph nodes (MLN) (**Figure 1D**) both in health and in disease as evidenced in translocation of enteric microorganisms to MLN in patients with advanced cirrhosis (prevalence 30.8%) and comparable prevalence of bacterial translocation (~8%) in patients and healthy controls after selective intestinal decontamination (100). Consequently, it is postulated that immune cells interacting with bacteria are activated in the MLN after which they are redistributed in the blood (**Figure 1H**) (101). Similarly, bacterial products have also been implicated as mediators between the gut microbiota and peripheral organs: A major part of consumed dietary fibers are fermented by bacteria in the colon, which results in various metabolites, including short chain fatty acids (SCFA) (102). SCFA are readily absorbed by the colonocytes and only a small part is secreted (103). Production of SCFA increases with a fiber-rich diet (102), and increased circulating levels of SCFA are associated with improvements in insulin sensitivity and decreased levels of lipolysis, triacylglycerols, and free fatty acids in humans (104).

Beyond the blood, there seem to be unexpected and promising niches of bacterial translocation and bacterial targeted action are currently drawing increasing multidisciplinary attention leading to a paradigm shift in the study and definition of metabolic diseases as "non-communicable" diseases.

Impact of Blood Borne Bacteria and Bacterial Components on Metabolic Disease

Lipopolysaccharides in Health and Disease

The measurement of bacterial components in the circulation allows an indirect verification of increased intestinal permeability. This link has emanated from the observation that sepsis is associated with an acute but reversible state of insulin resistance (105). To this end, most studies have focused on the measurement of LPS, i.e., endotoxins, which are molecules on the outer membrane of gram-negative bacteria. The condition of increased exposure to bacterial LPS in the blood in obesity or metabolic disease is referred to as "metabolic endotoxemia". Fat intake is associated with an increase in postprandial LPS levels (106), which have been shown to be distributed from the gut into the circulation *via* the mesenteric lymph nodes using freshly formed chylomicrons (**Figure 1D**) (107). LPS is recognized and bound by both LPS-binding protein (LBP) and soluble cluster of differentiation (sCD14), which can also be used as marker proteins, and recruited to the TLR 4 (108). The highest proportion of LPS in the circulation is bound to lipoproteins, whereby most of LPS is bound to high density lipoprotein (HDL) (109). Smaller fractions of LPS are also bound to platelets (110, 111), monocytes (112) and erythrocytes (113, 114) or are present as free LPS (115) (**Figure 1G**). However, these observations mostly stem from models of sepsis. Endotoxin and other bacterial components taken up from the gut due to increased

intestinal permeability will presumably enter the portal vein and, as a first step be transported to the liver, where they are rapidly cleared. Clearance mostly takes place in the liver after uptake *via* – among other routes – various lipoprotein receptors, e.g., LDL-R (115) and is mainly accomplished by hepatocytes and Kupffer cells to be then excreted to the bile (**Figure 1E**). Moreover, since binding of LPS with lipoproteins and chylomicrons has been shown to prevent endotoxin induced monocytes activation and secretion of proinflammatory cytokines (116, 117), the redistribution and increased content of phospholipids among the different lipoproteins has been suggested as a potential mechanism for the attenuation of the immunostimulatory effects of LPS.

LPS is detectable in low concentrations even in healthy subjects, and a single meal with high fat content already increases LPS levels (118, 119). In the last years, many studies in large cohorts reported increased levels of LPS and LBP in subjects with metabolic syndrome or T2D (120–123). Pussinen et al. analyzed LPS levels of patients with prevalent (n=537) and incident T2D (n=462) and compared them to a control group (n=6,170), of which about 20% had the metabolic syndrome. Endotoxin was significantly increased in individuals with T2D and endotoxin activity was associated with an increased risk for incident T2D, independently of other metabolic risk factors (121). Furthermore, serum endotoxin was linearly associated with the number of metabolic syndrome traits (121). Direct administration of LPS induced insulin resistance and systemic inflammation (124) and an 8-weeks long overfeeding intervention was associated with increased endotoxemia, linking overnutrition with endotoxemia and insulin resistance (125). A more recent study by Cox et al. used LPS, LBP as well as intestinal fatty acid binding protein (iFABP) to calculate a permeability risk score, which was increased in individuals with type 2 diabetes (123). In secondary complications, such as NAFLD, even higher endotoxin levels are reported (126). Mechanistic insights in the cascades underlying the effects of LPS on gut permeability and subsequent metabolic impact stem from mice studies, where subcutaneous LPS infusion has been shown to lead to an obese phenotype, comparable to that of mice on high-fat diet, including increased glucose and insulin levels as well as whole body and adipose tissue weight gain (106). Moreover, LPS application *in vitro* has been shown to result in an increase in gut permeability through tight junction dysfunction *via* a TLR4-dependent process (127), which is the most specific LPS receptor (128, 129) (**Figure 1I**).

As for the impacts of metabolic intervention on LPS: Roux-en-Y gastric bypass (RYGB) surgery has been shown to reduce LPS levels by 20 ± 5% after a 180 days follow-up period in patients with obesity and T2D (122), which could also be confirmed for sleeve gastrectomy and duodenal switch procedures (130, 131).

Most of the studies looking at LPS however neglect the fact, that LPS from different bacteria has a high heterogeneity in its chemical structure, mostly due to variation in the O-antigen polysaccharide (132, 133), and consequently, biological function (134). The latter was nicely demonstrated by Vatanen et al., showing that LPS derived from *B. dorei* counteracts the immunostimulatory activity of *E. coli* LPS (134). To our knowledge, there are no studies looking at LPS variation in metabolic disease published so far.

That being said, a reduction of intestinal permeability has been evidenced both in rat and human models of bariatric surgery (135). In rats undergoing duodenojejunal bypass surgery (DJB), intestinal permeability as assessed by Ussing chamber studies and dextran-FITC through mucosa-to-serosa flux was reduced in the alimentary and common limb as well as the colon, consistently supported by increased mucosal expression of occludin in both the alimentary and common limbs after DJB. LPS and LBP levels were not different between DJB and sham operated rats, possibly due to increased bacterial counts and compensatory increased intestinal surface in the remaining intestine. This is in contrast to data from humans, where mucosal surface was significantly reduced after surgery and occludin as well as zonula occludens expression was reduced. Congruently though, intestinal permeability as assessed by Ussing chambers was decreased owing to an increased expression of claudin-3-expression (136), further tying up reduction of microbiota-derived inflammatory mediators and rearrangement of gut barrier regulators after bariatric surgery (135).

Bacteria and Bacterial Fragments

In addition to LPS measurement, other bacterial components such as bacteria itself or bacterial DNA have been suggested as possible contributors to metabolic disease. The amplification of bacterial DNA allows quantification of bacterial load while subsequent sequencing allows the assessment of microbial composition and identification of specific bacterial perpetrators. There is increasing evidence for the presence of bacteria in blood, even in healthy subjects, as extensively discussed by Castillo et al. (100). Briefly, there are multiple studies reporting concurrent bacterial phyla in blood with Proteobacteria and Firmicutes being the most dominant phyla (100, 137). The first study to relate bacterial presence in the blood with metabolic disease was published by Amar et al. in 2011 and included subjects of the D.E.S.I.R. cohort within a longitudinal study aimed at understanding the complex pathophysiology of the metabolic syndrome (138, 139). 16S rRNA gene quantities were measured in 3,280 subjects at baseline and after nine years. Subjects who developed T2D during the duration of the study had significantly higher amounts of bacterial DNA at baseline (odds ratio=1.35, p=0.002). Moreover, bacterial DNA was significantly elevated in subjects with abdominal obesity (odds ratio=1.18, p=0.01). Additionally, pyrosequencing showed the dominant phylum to be Proteobacteria (139), whose amount was found to be an independent risk factor of cardiovascular disease in a subsequent analysis in 3,936 participants of the same cohort (140).

Similarly, blood bacterial DNA composition reminiscent of that observed for the gut microbiome was found in a Japanese cohort of 100 subjects including 50 with T2D. In addition, the detection rate for bacterial DNA was significantly higher in

subjects with T2D compared with healthy subjects (28% vs. 4%, p<0.01) (141). In line with above mentioned studies, patients with obesity and liver fibrosis seem to have elevated concentrations of bacterial DNA compared to patients with obesity alone (142). Most recently, Qiu et al. showed that subjects carrying *Bacteroides* had a reduced risk while patients carrying *Sediminibacterium* an increased risk to develop T2D (143). Lammers et al. analyzed the effect of bacterial DNA of eight probiotic strains including Bifidobacterium and Lactobacillus on peripheral blood mononuclear cells (PBMCs) of healthy donors. All strains increased expression of *IL-1, 6, and 10* at high concentrations of 70 µg/ml with largest changes observed for *B. infantis* showing that bacterial DNA can indeed induce strain specific immune alterations in the host (144).

In a small cohort of 58 subjects with obesity, who had undergone bariatric surgery, Ortiz et al. analyzed the translocation of bacterial DNA before and up to 12 months post-surgery. Bacterial DNA was detected in 32.8% of the patients prior to surgery, but only in 13.8% and 5.2% of patients after 3 and 12 months, respectively (145). More importantly, inflammation, LPS levels and insulin resistance persisted in subjects with a reduced clearance of bacterial DNA in the blood even after significant weight loss following bariatric surgery with multivariate analyses revealing bacterial DNA presence as an independent predictor (145). In summary, there is increasing evidence not only for the presence of bacterial DNA and potentially intact bacteria even in healthy subjects but also for the association between the amount of bacterial DNA and bacterial composition with obesity and related metabolic traits. However, current methods have considerable limitations (see *Limitations* of the review) and further, more elaborative studies are warranted to substantiate initial results.

Bacteria and Bacterial Products Regulate Adipose Tissue Inflammation Locally

The human adipose tissue is a metabolically active organ with large heterogeneity in cellular composition, function, and expression signatures depending on its anatomic location (146).

LPS, Adipocytes, and Adipose Tissue Macrophages

Although there are only a few studies providing evidence for the existence of bacterial components in adipose tissue, their potential impact has been repeatedly demonstrated in cell culture experiments. Toll-like receptors, especially TLR4, which are needed for the recognition of LPS are expressed on adipose tissue macrophages as well as adipocytes (147). Stimulation of adipose tissue macrophages with LPS induces fibrosis *via* TLR4 and the induction of the profibrotic factor Transforming growth factor beta 1 (TGFß1) (148). LPS can also act directly on adipocytes, as demonstrated in numerous studies (149–153). Creely et al. could show that IL-6 and TNF-α are secreted in response to LPS in mature subcutaneous adipocytes from lean individuals and subjects with obesity, whereas inhibition of NF-kB reduced the levels of secreted IL-6 after LPS stimulation (149). These results could be confirmed by Vitseva et al. using subcutaneous abdominal fat from subjects with obesity (150). Further works suggest a role for LPS on modifying glycerol permeability and metabolism of murine 3T3-L1 adipocytes (153) and hint to a role of adiponectin in regulating local inflammation by inhibiting LPS-induced NF-kB activation using primary pig adipocytes (152).

Bacterial DNA and Live Bacteria

First evidence that bacteria are indeed able to translocate from the gut to adipose tissue stems from mouse experiments. In a pioneering work, Amar et al. used, among other methods, green fluorescent protein (GFP)-labeled *E. coli* to demonstrate the transmucosal passage in mice on high-fat diet (154). Although some initial studies suggested the presence of bacterial DNA in human adipose tissue as well (155), others could not confirm these results (156). In their work, Zulian et al. isolated DNA from whole adipose tissue and mature adipocytes of 14 subjects with obesity. Bacterial PCR products were only observed in the enzymatically isolated adipocytes, but sequencing revealed that 90% of this bacterial DNA belonged to *C. histolyticum*, from which the collagenase enzyme used for adipocytes isolation is derived. All subsequent experiments including culturing experiments were negative (156). In 2017, Udayappan et al. reported the presence of bacterial DNA, mainly stemming from *Actinobacter* and *Ralstonia*, in mesenteric adipose tissue of twelve patients with obesity. However, as they did not include negative controls, contamination as a source of these observed genera could not be excluded (157). Another noteworthy study published by Nakatsuji et al., investigated the subepidermal adipose tissue of six healthy subjects and suggested that the skin microbiome extends to this specific skin-adjacent adipose tissue depot (158). Furthermore, bacterial DNA belonging to several bacteria was observed in the epicardial adipose tissue in six patients with acute coronary syndrome (*Streptophyta* and *Rickettsiale*) and stable angina (*Pseudomonas* and *Moracellaceae*) as compared to subjects with isolated mitral insufficiency. The authors related coronary heart disease with increased bacterial colonization of epicardial adipose tissue and ensuing inflammasone activation (159). More recently, Anhê et al. reported data on contaminant-controlled presence of bacterial DNA in mesenteric, subcutaneous and visceral adipose as well as liver tissue in 40 patients with obesity. Hereby, highest abundance was observed in visceral adipose tissue and liver samples, and patients with T2D had a decreased bacterial diversity. Diversity was especially high in patients with obesity but without T2D in mesenteric adipose tissue (160). Data recently presented by our group could extend and go beyond the proof of bacterial DNA by showing the presence of living bacteria in adipose tissue using catalyzed reporter deposition (CARD) - fluorescence *in situ* hybridization (FISH) methods. Furthermore 16S rRNA gene content of 75 patients with obesity was quantified and sequenced, showing that both bacterial quantity and taxonomy were associated with markers of inflammation and insulin resistance (137). Moreover, miniscule amounts of bacterial DNA (*E. coli*) were sufficient to induce a proinflammatory response in human subcutaneous

adipocytes, as observed by a dose-dependent increase of *IL6* and *TNF* expression. Unexpectedly, expression of neither *TLR4* and *TLR9*, nor the NLR family pyrin domain containing 3 (*NLRP3)* inflammasome increased at any concentration (137). Similar direct evidence for the translocation of *via*ble gut bacteria to adipose tissue, in this case mesenteric adipose tissue, was presented by Ha et al. using human biopsies, gnobiotic mice as well as primary cells (161). Bacterial translocation occurred in both healthy subjects and patients with Crohn`s disease (CD), but taxonomic profiles differed. Using gnobiotic mice treated with *C. innocuum*, the bacterium most frequently isolated in CD, Ha et al. evidenced translocation of these bacteria into adipose tissue. This was accompanied by increases in *adiponectin* and Peroxisome proliferator-activated receptor Gamma (*PPARG)* expression and adipose tissue expansion. These results suggest that, at least in the case of CD, specific bacteria including *C. innocuum* can restructure mesenteric adipose tissue leading to the expansion and fibrosis of creeping fat. This might be a defense mechanism to prevent systemic translocation of bacteria beyond this compartment (161).

Impact of Bacterial Metabolites on Adipose Tissue Function

SCFA are recognized by multiple G-protein coupled receptors (GPR), and receptors GPR41, 43 and 81 are known to be expressed in adipose tissue (162). First evidence for an adipose tissue specific effect of SCFA was observed by Kimura et al., who showed that mice with a global GPR43 knockout were obese compared to wild type controls whereas mice with a selective overexpression of GPR43 in adipose tissue were leaner (163). The authors further demonstrated that GPR43 stimulation with acetate improved glucose and lipid metabolism in adipose tissue, but not in muscle and liver (163), thereby confirming prior results by Robertson et al., that showed reduced lipolysis in adipose tissue after stimulation of GPR43 with acetate (164). These results were recently strengthened by experimental treatment of multipotent human adipose tissue-derived stem cells with either mixtures of butyrate, propionate and acetate or the individual SCFA in varying concentration between 1 µmol/l and 1 mmol/l. Whereas mixtures high in butyrate had no effect on glycerol release, mixture with high concentration of acetate and propionate decreased basal glycerol release (165). Treatment with only butyrate even increased glycerol release slightly but significantly, whereas isolated treatment with acetate reduced glycerol release (165). Next to their effects on lipolysis, acetate and propionate also seem to influence adipogenesis *via* GPR43, as shown by Hong et al. in 3T3-L1 cells (166). The authors found no GPR43 expression in the stromal vascular fraction (SVF) or preadipocytes, but expression levels increased gradually with adipogenesis. Furthermore, siRNA mediated silencing of GPR43 blocked adipocyte differentiation (166). A further study analyzed the effect of SCFA-rich HFD compared to normal HFD diet on adipose tissue biology: Supplementation of SCFA increased expression of GPR43 in adipose tissue and reduced its expression in the colon. Next to decreased leptin and increased adiponectin expression in adipose tissue, the authors showed an increase in adipose tissue beigeing under SCFA treatment (167).

Pancreas and Liver: Bacterial Actions on Central Metabolic Organs

LPS Effects on Liver and Pancreas

The liver has been shown to participate in active LPS clearance. Increased amounts of endotoxin are still not without consequence for the liver: As a response to LPS uptake and stimulation, hepatocytes express acute phase protein serum amyloid A, which further promotes LPS clearance (168). However, hepatocytes can also utilize the TLR4 cascade to take up LPS (169, 170). That being said, it is assumed that altered TLR4 signaling is a key factor in metabolic liver diseases including NAFLD (171). TLR4 is expressed on various liver cell types including hepatocytes, monocytes, Kupffer cells and stellate cells (172). Using primary human liver biopsies and human hepatocytes (HepaRG), it was shown that LPS increases NF-kB translocation and activity by ~2.5 fold, although the observed effect was only partially mediated by TLR4 as demonstrated by siRNA knockdown and chemical blocking of TLR4 (173). Kheder et al. stimulated macrophages (J774) and HepG2 hepatocytes with LPS, which led to increased TNF-α expression (**Figure 1E**). Co-treatment with increasing doses of vitamin D3 and docosahexaenoic acid (DHA) reduced these effects in a dose dependent manner (174). Lee et al. used mouse hepatocytes deficient for TLR4, Myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) to show that LPS induces hepcidin expression, an antimicrobial and iron regulating protein, *via* TLR4 in a MyD88 dependent manner (175).

On the other hand, endotoxins have been shown to exert varying effects on various pancreatic cells: Endocrine cells of the human pancreatic islets express CD14 and TLR4. CD14 was synthesized and secreted by SV40-transformed islet cells (HP62) after treatment with LPS. LPS was shown to regulate glucose-dependent insulin secretion and induced an inflammatory response (176). By analyzing isolated human and rodent islets, Amyot et al. showed that LPS impairs insulin expression, whereby human islets are more sensitive to this effect. LPS further decreases the expression of pancreatic and duodenal homeobox 1 (PDX-1) and Transcription factor Maf (MafA) and this inhibition is prevented by blocking NF-kB but not p38 MAPK signaling, linking bacterial induced inflammation with pancreatic dysfunction (177).

Bacterial Translocation to the Liver

Bacterial DNA has also been evidenced in liver tissue under contamination control (160). More recently, Sookoian et al. were able to localize LPS derived from Gram-negative bacteria in the portal tract and evidenced a diverse repertoire of bacterial DNA in the liver, the composition of which was driven in part by obesity. This metataxonomic signature was furthermore related to histological findings in NAFLD, with expansion of proteobacterial DNA being associated with lobular and portal inflammation as well as liver cirrhosis in non-morbidly obese

subjects. Morbid obesity on the other hand displayed wider associations to several taxa thought to contribute to more detrimental findings (178).

SCFA Impact on Liver Function and Pancreas

Most of the SCFA taken up from the colon are metabolized by the liver and only small proportions enter the peripheral circulation (about 20% for butyrate and propionate) (179–181). Treatment of HepG2 cells with SCFA ranging from C3 to C6 increased Apolipoprotein A (ApoA-I) expression in a dose-dependent matter whereas secreted ApoA-I to medium was reduced. Additionally, butyric acid increased carnitine palmitoyltransferase I (CPT1) expression, a key protein of beta-oxidation, and increased activity of PPARA (180). Butyrate was also shown to improve insulin sensitivity and increase energy expenditure in mice, and mice on HFD treated with butyrate exhibited higher protein levels of cyclic 5' AMP-activated protein kinase (cAMPK), P38 and PGC-1a in the liver compared to HFD mice without treatment (182). Sahuri-Arisoylu et al. analyzed the effect of acetate, showing that acetate decreased lipid accumulation and improved hepatic function. The authors observed decreased levels of circulating aspartate transaminase and alkaline phosphatase, as well as reduced expression of genes involved in lipogenesis, such as fatty acid synthetase (183). Acetate also protects rodent models against diet-induced weight increase by altering liver metabolism, as Kondo et al. showed by increased expression levels of fatty acid oxidation enzymes as well as PPARA in the liver (184).

As in adipose tissue, GPR43 is also expressed on pancreatic β-cells, and expression is increased in mice fed with HFD. GPR43 knockout in mice leads to impaired insulin secretion and treatment of murine and human islet cells with a GPR43 agonist leads to increased intracellular Ca2+ and inositol triphosphate levels as well as increased insulin secretion (185). In contrast, a study concurrently did not evidence any effects on glucose homeostasis in GPR43-/- mice on normal chow or HFD compared to wild type mice. In *ex vivo* studies, presence of acetate potentiated insulin secretion (186). Similarly, it was shown that propionate potentiated dynamic glucose-stimulated insulin secretion *in vitro* and further protected human islets from inflammatory cytokine and sodium palmitate induced apoptosis (179).

Impact of Bacteria and Bacterial Products on Skeletal Muscle Metabolism

Evidence on the role of endotoxin on muscle tissue insulin sensitivity mainly stems from septic models. During hyperinsulinemic euglycemic clamps, 4 ng LPS per kg body weight were administered to healthy donors, resulting in an increased peripheral glucose uptake as well as increased circulating concentrations of norepinephrine and cytokines (180). Concurrently Reyna et al. evidenced an elevated TLR4 expression in muscle tissue of subjects with obesity and T2D and concentrations of TLR4 protein isolated from muscle tissue correlated positively with insulin resistance (181). Furthermore, patients exhibited lower IκBα content and increased expression of *IL6* and *SOD2* and these results could be replicated in primary human myotubes by treatment with palmitate (181). More functional approaches were, among other performed by Liang et al., who similarly differentiated primary human muscle cells into myotubes and treated them with LPS. Endotoxin induced inflammation and reduced insulin signaling; effects which were counteracted by a treatment with the TLR4-Inhibitor (182). These results are supported by data published by Frisard et al. on treatment of mice and human myotubes with high and low concentrations of LPS, thereby reflecting septic or metabolic endotoxemia conditions and showing an increased glucose utilization and reduced fatty acid oxidation in skeletal muscle (183). Low levels of endotoxin are sufficient to modulate mitochondrial consumption and substrate oxidation preferences, an effect which was absent in the presence of specific antioxidants, thereby suggesting a role of reactive oxygen species in mediating observed effects (184).

Houghton et al. tested the effect of various microbiome catabolites, including phenolic compounds, bacterial metabolites, and their phenolic conjugates. Several of these compounds increased glucose uptake to differentiated human skeletal myoblasts (LHCN-M2) as well as overall metabolism rates (187). Most prominent effects were observed for isovanillic acid 3-O-sulfate (IVAS) and dihydroferulic acid 4-O-sulfate. IVAS furthermore promoted upregulation of GLUT1, GLUT4, and phosphoinositid-3-kinase (Pi3K) p85α proteins (187).

The effect of SCFA on skeletal muscle metabolism and function was recently reviewed in an excellent publication by Frampton et al. (188). Studies examining direct effect on skeletal muscle cells are mainly using rat-derived L6 myotubes or mouse-derived C2C12 myotubes and no human studies were found. Briefly, treatment with SCFA increased fatty acid oxidation (about 30% for 0.5 mM butyrate (189)) and fatty acid uptake (i.e., using 0.5 mM acetate) in L6 myotubes (190). Furthermore, stimulation with acetate and propionate promotes enhanced insulin-independent uptake of glucose to both mouse and rat myotubes (190, 191). Maruta et al. also report an upregulation of GLUT4 gene expression as well as protein levels upon treatment with 0.5 mM acetate (190).

LIMITATIONS

It can be assumed that subjects not suffering from sepsis have lower bacterial load and display lower endotoxemia making careful handling of samples to avoid contamination, careful data interpretation to avoid over-reporting of false positive results as well as method standardization allowing comparability and reproducibility, paramount aspects for planning studies contributing to the subject as well as interpretation of the available literature. Here, we highlight some concerns, which should be considered in interpretation of relevant literature.

Measurement of Intestinal Permeability

There are various methods to measure the integrity of the intestinal barrier with differing degrees of sensitivity and specificity: Methods include Ussing chambers (87), histology

and electron microscopy for biopsies and transepithelial/transendothelial electrical resistance (TEER) for cell culture experiments (192). The gold standard for *in vivo* studies is the lactulose/mannitol (La/Ma) or similar dual sugar tests, which assess the flow of indigestible sugars from gastrointestinal tract to the circulation and the combination of different sugars allows a location specific measurement (34, 193). In addition, biomarkers such as calprotectin, alpha-1-trypsin, fatty acid binding protein, and zonulin are used. There is extensive literature on benefits and disadvantages of each method (34, 193–196). However, in our opinion, studies employing biomarkers are generally lacking in strength due to various reasons: a) correlations with dynamic permeability tests such as dual sugar tests or Ussing chambers are not present or very weak; b) most of these markers are also acute phase proteins and are therefore increased in inflammation; c) their actions are not limited to the gut paracellular pathway; d) commercially available ELISAs are often not validated, as we and others recently demonstrated that the preferred ELISA for the most commonly used biomarker zonulin measures different products (197, 198). Consequently, relevant literature should be interpreted carefully in this regard (199).

Endotoxin

Measurement of endotoxin, in particular at low concentrations, is very challenging and prone to errors, leading to considerable discussion pertaining to the significance of the test and its interpretation. One major aspect to be considered is that LPS are not actively excreted by bacteria but that release of LPS only takes place after gram negative bacterial cell death and lysis, which in turn does not justify the wide use of LPS as a surrogate marker for "live" bacterial translocation. However, there could still be an intrinsic value for the increased exposition of the host to bacteria in general. Measurement of LPS has routinely been done using the Limulus Amoebocyte Lysate (LAL) assay. Although the FDA has recognized the validity of approaches using the LAL method, LAL reagents are not required to be obtained from an FDA-licensed manufacturer when used in the context of research, making standardization between methods reported in the literature almost impossible. Beyond this, conducting the LAL assay is highly challenging. This is related to the magnitude of chemical and physical products and factors, which are known to interfere with the test's ability to detect LPS. These factors include pre-analytical conditions such as sampling, where plastic or siliconized ware can lead to endotoxin absorption (200) and render LAL assay ineffective. Moreover LPS quantification presumes sampling in pyrogen/LPS free ware, which can be done *via* dry heat sterilization at high temperature, a widely ignored factor possibly contributing to the immense range of LPS from 0.01 to 60 EU/ml reported in healthy subjects in the literature (120, 201). Furthermore, various factors within the blood can interfere with LPS testing, including bile salts, lipoproteins, EDTA and heparin (202), which makes pretreatments of samples to measure LPS unavoidable. Moreover, using different units impedes the interpretation of findings from different studies (203): whereas some studies report LPS levels in weight per water volume (pg/mL), others report endotoxin units per unit volume (EU/mL), which reflects LPS activity. Additionally, when considering that host-derived proteins binding of LPS largely modulates its activity and clearance from the circulation (204–206), it becomes evident that whatever is measured after pretreatment of the samples to overcome "low LPS recovery" might not reflect *in vivo* conditions at all. This is best exemplified by the fact that there is poor concordance between endotoxemia and gram-negative bacteremia and that endotoxemia is detected in less than 50% of subjects with gram-negative sepsis (207). Independent from units, most studies reported a 0.5 to 2-fold increase of LPS in subjects with obesity compared to subjects with normal weight, as clarified by Boutagy et al. (203). Consequently, they propose using fold changes (in disease vs. controls) instead of absolute values to describe the contribution of endotoxemia to the study setting until unified and comparable methods are established.

In addition, LPS derived from different bacteria can show large heterogeneity in the O-antigen polysaccharide with consequence on the inflammatory potential and biological function of endotoxin (133, 134). This point is normally neglected in studies on endotoxin and should therefore be considered more often in new studies.

Bacterial DNA

In studies analyzing bacterial DNA in samples with low bacterial biomass, be it quantification or sequencing, contamination is a major problem, which is well-highlighted by the recent debate about a possible placenta microbiome (208–211). Contamination can arise from multiple sources (air, skin, reagents) and during all experimental steps. Furthermore, even sterile lab ware can contain traces of bacterial DNA, as per definition a sterile environment is only defined by the absence of living microorganisms (212). The use of adequate negative controls and the deployment of subsequent bioinformatic tools to address them become eminent to avoid reporting false positive results (213). Although these problems are increasingly addressed by the research community, a set of consistent and widely accepted approaches would help for the general comparability and reproducibility of data.

Data Interpretation

In most works on metabolic endotoxemia it is assumed that LPS is the consequence of impaired intestinal barrier. However, other locations can contribute to a metabolic endotoxemia as well. For instance, it was shown that oral interventions like extraction of teeth or periodontal probing but also everyday actions such as chewing and oral hygiene can lead to an influx of endotoxin and bacteria into the circulation (214, 215). It was also observed that subjects with obesity are more prone to suffer from gingivitis, which is most likely due to increased insulin resistance (216, 217). It is therefore likely that LPS and bacteria from the oral cavity contribute more to systemic 'bacterial burden' and possibly inflammation than has been previously assumed. Additionally, the skin microbiome extends to various compartments, thus implicating a potential impact for this compartment in translocation to subsequent tissues (158). However further studies are desirable to support these findings. Furthermore, since LPS is rapidly cleared from the circulation in

the liver, which is also the first organ reached by LPS taken up from the gastrointestinal tract, whatever measureable endotoxin under various conditions points toward a chronic influx and the additional contribution of other origins like oral cavity, lungs, or the skin (**Figure 1F**).

SUMMARY/CONCLUSION

In this review, we focused on causes and possible consequences of impaired intestinal permeability, thereby focusing on obesity and components of metabolic diseases. Consequences include translocation of endotoxin, bacterial DNA, or live bacteria as well as bacterial metabolites to the circulation, a process often associated with the term metabolic endotoxemia or bacteremia. Starting with animal studies, there now is also compelling evidence for an impairment of the human intestinal barrier in diseases such as T2D and obesity. Consequently, increased circulating bacterial load is now a well-established hallmark of metabolic diseases and published data suggest that impairment of the gut barrier can trigger and further aggravate metabolic impairment. A less studied subject is the presence and effect of bacteria in other host tissues, including liver, muscle, pancreas, and adipose tissue.

However, as shown in this review, several studies suggest that tissue bacteria or components and metabolites thereof either reflect or directly contribute to the development and progression of metabolic diseases. Underlying mechanisms such as TLR4 dependent activation of NF-kB have been introduced but a holistic approach encompassing the complexity of host immune factors is widely lacking. There has also been compelling underreporting of shortcomings in the methods used such as LPS measurement, making claims toward causality rather elusive. Furthermore, only a few studies were published on quantification and sequencing of bacterial DNA in host tissues, which have been similarly plagued with false positive results partly due to contamination.

In conclusion, it seems unavoidable that our bacterial inhabitants also contribute to the modulation of their metabolic environment shaping the body's responses to nutrients and contributing ultimately to disease as has been shown for the gut microbiota in recent years (218). Considering the current relevance of microbiome research in understanding health and nutrition and the promising avenues for therapy and prevention, it will be inevitable to revisit many of the notions introduced here to allow for robust and reliable mechanistic approaches.

AUTHOR CONTRIBUTIONS

LM, PK, and RC designed the review. LM and RC interpreted literature and drafted the manuscript. LM designed the figure. MB and PK critically reviewed and edited the manuscript. All the authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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GLOSSARY

TLR	toll-like receptor
T2D	type 2 diabetes
TJ	tight junctions
IgA	immunoglobulin A
PKC	protein kinase C
MAPK	mitogen-activated protein kinase
INF-γ	interferon gamma
TNF-α	tumor necrosis factor alpha
PI-3K/Akt	Phosphoinositide 3-kinases/Protein kinase B pathway
NfkB	nuclear factor kappa-light-chain-enhancer of activated B cells
ERK	extracellular signal-regulated kinase
ARF6	ADP-ribolysation factor-6
HIF-1α	Hypoxia-inducible factor 1-alpha
GLUT1; 2; 4	glucose transporter
Cxcr	CXC-Motiv-Chemokine receptor
HFD	high fat diet
db/db	leptin receptor deficient mouse model
ob/ob	leptin gene overexpression mouse model
ZO	zonula occludens
PAI-1	Plasminogen-Aktivator-Inhibitor 1
STAMP2	six transmembrane protein of prostate 2
IL-1, 6, 10	interleukin 1, 6, 10
La/Ma	lactulose mannitol
HOMA-IR	homeostatic model assessment for insulin resistance
NAFLD	Non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
MLN	mesenteric lymph nodes
SCFA	short chain fatty acids
LPS	lipopolysaccharide
sCD14	soluble cluster of differentiation 14
LDL	low-density lipoprotein
HDL	high-density lipoprotein
LDL-R	LDL receptor
iFABP	as intestinal fatty acid binding protein
LBP	LPS binding protein
DJB	duodenojejunal bypass surgery
FITC	Fluorescein isothiocyanate
TGF-β1	Transforming growth factor beta 1
GFP	green fluorescent protein
CARD-FISH	catalyzed reporter deposition fluorescence *in situ* hybridization
NLRP3	NLR family pyrin domain containing 3
PPARG	Peroxisome proliferator-activated receptor gamma
CD	Crohn's disease
GPR	G protein-coupled receptor
SVF	stroma vascular fraction
DHA	docosahexaenoic acid
MyD88	Myeloid differentiation primary response 88
TRIF	TIR-domain-containing adapter-inducing interferon-β
PDX-1	pancreatic and duodenal homeobox 1
MafA	Transcription factor Maf
cAMPK	cyclic 5' AMP-activated protein kinase
C3	complement component C3
ApoA1	Apolipoprotein A1
SOD2	Superoxide dismutase 2
mitochondrial, IVAS	isovanillic acid 3-O-sulfate
ELISA	enzyme-linked immunosorbent assay
LAL	Limulus Amoebocyte Lysate
EU/ml	endotoxin units per ml
FDA	United States Food and Drug Administration

**Terms are ordered as they appear in the text for the first time.*

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REVIEW
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Modulating the Microbiota as a Therapeutic Intervention for Type 2 Diabetes

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Mounting evidence suggested that the gut microbiota has a significant role in the metabolism and disease status of the host. In particular, Type 2 Diabetes (T2D), which has a complex etiology that includes obesity and chronic low-grade inflammation, is modulated by the gut microbiota and microbial metabolites. Current literature supports that unbalanced gut microbial composition (dysbiosis) is a risk factor for T2D. In this review, we critically summarize the recent findings regarding the role of gut microbiota in T2D. Beyond these associative studies, we focus on the causal relationship between microbiota and T2D established using fecal microbiota transplantation (FMT) or probiotic supplementation, and the potential underlying mechanisms such as byproducts of microbial metabolism. These microbial metabolites are small molecules that establish communication between microbiota and host cells. We critically summarize the associations between T2D and microbial metabolites such as short-chain fatty acids (SCFAs) and trimethylamine N-Oxide (TMAO). Additionally, we comment on how host genetic architecture and the epigenome influence the microbial composition and thus how the gut microbiota may explain part of the missing heritability of T2D found by GWAS analysis. We also discuss future directions in this field and how approaches such as FMT, prebiotics, and probiotics supplementation are being considered as potential therapeutics for T2D.

Keywords: microbiota (16S), type 2 diabetes (T2D), metabolites, probiotics, prebioitcs, intermittent fasting, genetics, epigenetics

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INTRODUCTION

Diabetes is a metabolic disorder characterized by elevated blood glucose levels. The incidence of diabetes is widespread and the International Diabetes Federation (IDF) reports that 463 million people in the world are suffering from diabetes, which is estimated to reach 700 million by the year 2045 (1). In the USA, 13% of adults aged 18 or older have diabetes (2). Diabetes has been categorized into three classes (type 1, type 2, and gestational diabetes) depending on the underlying pathophysiology. Among them, type 2 diabetes (T2D) accounts for more than 90% of all diabetes (1, 2). Though genetic susceptibility is a critical determinant of T2D, non-genetic

factors, including diet, physical activity, also play a significant role in the development and severity of T2D. The recent understanding that microbiota is a critical determinant of human health has opened a new avenue of basic and clinical research for T2D.

The microbiota refers to an assemblage of living microorganisms including bacteria, archaea, and fungi present in a defined environment (3). The microbiota can reside within or on the host and can modulate nutritional status, health, and diseases of the host (4). The most widely used technique to assay microbiota is to quantify the variable region(s) of the 16S rRNA gene (5). 16S rRNA gene analysis is economical and straightforward, however, it provides a limited regulation of taxonomic information, up to the genus level reliably. Alternatively, metagenomics, a whole-genome shotgun sequencing approach of all the DNA present in the sample, provides much better taxonomic resolution down to species or strain level. Additionally, metagenomics encompasses the collective functional genomes of all microorganisms, thus provides an opportunity for functional profiling of the metabolic pathways present in a community (6). The diversity and composition of the gut bacteria have been intensely studied, as well as their impact on health and diseases (7), including obesity (8), inflammation (9), and T2D (10). A better understanding of the link between the gut microbiota and metabolic disorders, especially T2D, may lead to advances in current treatment approaches, accurate disease monitoring, and development of novel therapeutics.

In addition to sex and age, both diet and the immune system contribute to the composition of the microbiota (11). The underlying architecture of the host's genetics may also shape the community structure of the gut microbiota (12). Several genetic variants are associated with T2D susceptibility (13) and it is speculated that a part of "missing heritability" described in genome-wide association study (GWAS) studies (14) may be explained by gut microbiota. Moreover, growing evidence suggests that gut microbial metabolites regulate gene expression through a variety of classic signaling pathways (14) and more recently epigenetics (15). Thus, understanding the complex interactions between microbiome, microbial metabolome, and host genome will assist the development of novel therapeutics.

In this review, we critically summarize the recent developments describing the role of microbiota on T2D susceptibility, development, and severity. In particular, we focus on the underlying biochemical mechanisms by which gut microbiota may affect T2D. A number of these mechanisms may be mediated by the host genetics, and epigenetics thus may be viable targets for precision medicine. The potential effects of prebiotics, probiotics, medication, and intermittent fasting on the microbiota and T2D are extensively discussed. Finally, we comment on the future direction in this field.

DYSBIOSIS, OBESITY, LOW-GRADE INFLAMMATION AND T2D

A growing number of studies suggest that gut microbiota influences T2D susceptibility, development, severity, and progression. Dysbiosis, an alteration of a healthy microbiota, is associated with obesity, low-grade inflammation, insulin resistance, and T2D which potentially reflects a causal role linking these pathologies (16). Along with animal studies, numerous human cohorts also have reported specific gut bacteria enriched or depleted in T2D patients compared to healthy controls. A summary of the recent reports of altered microbiota found in T2D patients is depicted in **Table 1**, and the interactions between environmental factors, genetics, microbiota, microbial metabolites, obesity, inflammation, and T2D are shown in **Figure 1**.

Landmark studies in the 2000s (27–29) demonstrated that the microbiota contributes to digestion, carbohydrate metabolism, obesity, and plasma glucose levels. Additionally, those studies established a causal relationship by showing that the susceptibility to obesity could be transferred between mice when the fecal microbiota of obese mice was transplanted into non-obese animals. Consistent with these findings, several other studies have reported enrichment or depletion of specific obesity-related gut bacteria and indicated a connection between gut microbiota, adiposity, and T2D. For example, increased abundance of *Prevotella* and decreased abundance of *Bacteroides* were associated with a higher risk of obesity with metabolic syndrome, while body mass index and body fat percentage were negatively correlated with *Coprococcus* abundance (30). More recent data suggests that the abundance of a bacteria in the Bacilli family was positively associated with fat mass, and negatively associated with lean mass and plasma glucose level (31). Additionally, *Peptostreptococcaceae*, *Blautia*, and a bacterium related to the *Clostridiaceae* family were positively associated with plasma glucose levels (31). In a recent randomized, double-blind, placebo-controlled clinical trial with overweight or obese insulin-resistant subjects, pasteurized *Akkermansia muciniphila* supplementation was associated with weight loss, improved insulin sensitivity, and reduced insulinemia (32). A potential mechanism of these positive effects is an interaction between temperature stable outer membrane protein Amuc 1100 found in pasteurized *Akkermansia muciniphila* and Toll-like receptor 2 (33).

Obesity and dysbiosis may cause low-grade inflammation (**Figure 2**) which also contributes to insulin resistance and the development of T2D. Several studies have demonstrated associations between gut microbiota, or microbial components, and low-grade inflammation in T2D (34). An array of bacterial components such as lipopolysaccharides (LPS) (35), flagellin (36), and peptidoglycan (37) can elicit an inflammatory response. LPS binds to immune cell receptors such as Toll-like receptors and Nucleotide Oligomerization Domain (NOD)-like receptors and triggers the expression of proinflammatory mediators that fuel chronic inflammation, promoting metabolic dysregulation and development of T2D (38). The interaction of specific microbes in the gut with the immune system is complex. Some gut bacteria and microbial components promote low-grade inflammation, while others stimulate anti-inflammatory cytokines and chemokines. For example, induction of interleukin (IL)-10 and IL-22 by species of

TABLE 1 | T2D-related gut microbiota found in human studies.

Sample size	Age	Sex	Technique	Associated microbiota changes	References
183 T2D 185 Controls (Chinese)	13–86	Women (153) Men (209)	Metagenomic sequencing	Increased in T2D: *Akkermansia muciniphila*, *Bacteroides caccae, Clostridium hathewayi, Clostridium ramosum, Clostridium symbiosum, Desulfovibrio* sp., *Eggerthella lenta*, and *Escherichia coli* Decreased in T2D: *Clostridiales* sp. *SS3/4, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis*, and *Roseburia inulinivorans* **Significantly correlated bacteria with T2D related traits: *Roseburia intestinalis* (-), *Faecalibacterium prausnitzii* (-), *Akkermansia muciniphila* (-), *Desulfovibrio* (-), *Bacteroides caccae* (+).**	Qin et al. (17)
53 T2D 49 Impaired glucose tolerance 43 Controls (Swedish females)	69–72	Women (145)	Metagenomic sequencing	Increased in T2D: *Clostridium clostridioforme, Lactobacillus gasseri*, and *Streptococcus mutans* Decreased in T2D: *Roseburia, Clostridium* spp., *Eubacterium eligens*, and *Bacteroides intestinalis* **Significantly correlated bacteria with T2D related traits: *Roseburia intestinalis* (-), *Faecalibacterium prausnitzii* (-), *Akkermansia muciniphila* (-), *Bacteroides intestinalis* (-), *Clostridium clostridioforme* (+), *Lactobacillus gasseri* (+).**	Karlsson et al. (18)
75 T2D, 291 Controls (Danish)	50–66	Women (187) Men (179)	Metagenomic sequencing	Increased in T2D: BCAA-producing bacteria *(Prevotella copri and Bacteroides vulgatus)* Decreased in T2D: *Faecalibacterium, Oscillibacter, Roseburia, Bifidobacterium, Coprococcus*, and *Butyrivibrio* **Significantly correlated bacteria with T2D related traits: *Faecalibacterium prausnitzii* (-), *Akkermansia muciniphila* (-), *Bacteroides vulgatus* (+), *Prevotella copri* (+), and *Clostridia* sp.**	Pedersen et al. (19)
46 T2D, 75 Combined glucose intolerance 178 Impaired glucose tolerance 189 Impaired fasting glucose 523 Controls (Swedish)	57–61	Women (568) Men (443)	Metagenomic sequencing	Increased in T2D: *Coprococcus eutactus, Clostridiales bacterium*, and *Lachnospiraceae bacterium* Decreased in T2D: *Clostridium* sp., *Clostridium hathewayi, Clostridium bolteae, Clostridium symbiosum*, and *Roseburia faecis*	Wu et al. (20)
13 T2D, 64 Prediabetes 44 Controls (Chinese)	52–55	NA	16S rRNA V3-V5 region	Increased in T2D: *Clostridia, Collinsella, Dorea, Prevotella, Ruminococcus*, and *Verrucomicrobia* Decreased in T2D: *Bacteroides, Akkermansia muciniphila, Faecalibacterium prausnitzii, Roseburia*, and *Streptococcus*	Zhang et al. (21)
20 T2D, 40 Controls (Chinese)	NA	Women (42) Men (18)	16S rRNA V4-V5 region	Increased in T2D: *Streptococcus, Dorea*, and *Fusobacterium* Decreased in T2D: *Akkermansia, Bifidobacterium, Faecalibacterium*, and *Parabacteroides*	Li et al. (22)
98 T2D, 193 Controls (Nigerian)	41–70	NA	16S rRNA V4 region	Increased in T2D: *Bacteroidetes, Prevotella, Desulfovibrio piger, Eubacteriu*, and *Peptostreptococcus* Decreased in T2D: *Anaerostipes, Ruminococcus, Cellulosilyticum ruminicola, Clostridium paraputrificum, Clostridium butyricum, Collinsella*, and *Epulopiscium*	Doumatey et al. (23)
18 T2D, 18 Controls (Danish males)	31–73	Men (36)	16S rRNA V4 region	Increased in T2D: Betaproteobacteria Decreased in T2D: Firmicutes and Clostridia	Larsen et al. (24)
134 T2D, 37 Controls (Chinese)	45–67	Women (92) Men (79)	16S rRNA V3-V4 region	Increased in T2D: *Prevotella, Dialister*, and *Sutterella* Decreased in T2D: *Bacteroides, Bifidobacterium, Clostridium XIVa, Parabacteroides, Staphylococcus, Granulicatella, Porphyromonas, Clostridium XI, Blautia, Anaerostipes, Clostridium XVIII, Fusicatenibacter, Enterococcus, Clostridium IV, Eggerthella*, and *Flavonifractor.*	Wang et al. (25)
22 T1D, 23 T2D, 23 Controls (Polish)	20–65	Women (40) Men (28)	16S rRNA	Increased: Firmicutes/Bacteroidetes ratio, Verrucomicrobia, Ruminococcus Decreased: *Bacteroides*, *Roseburia* and *Faecalibacterium*	Salamon et al. (26)

Roseburia, *Bacteroides*, *Akkermansia*, and *Lactobacillus* (33, 39–43) may contribute to restoring insulin sensitivity and improving glucose metabolism (43, 44). Similarly, *Bacteroides thetaiotaomicron*, *Roseburia intestinalis*, *Clostridium* clusters *IV*, and *XIVa* induce T_{reg} cells (45, 46), which are tolerogenic immune cells and are important for maintaining a balance between pro and anti-inflammatory immune responses (47). Additionally, butyrate produced by the gut microbiota enhances colonic T_{reg} differentiation through epigenetic modification of histone deacetylase inhibition (48, 49) and is discussed in detail below along with other short-chain fatty acids (SCFAs). Inhibition of pro-inflammatory cytokines and chemokines is another pathway that beneficial microbes use to prevent low-grade inflammation. Various species of

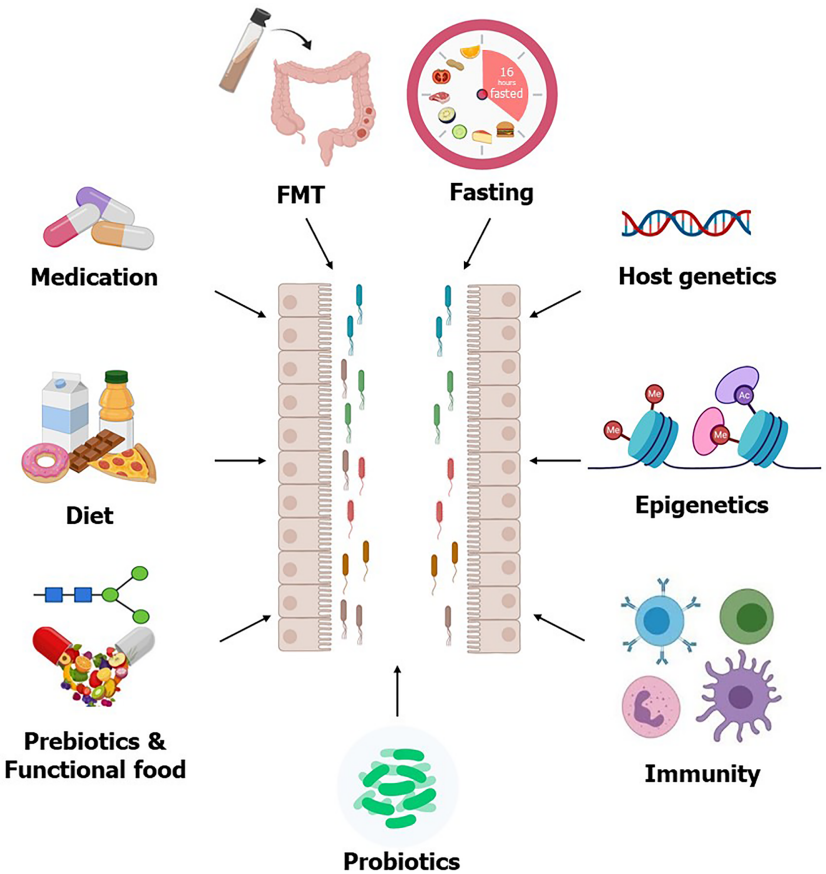

FIGURE 1 | **Factors affecting gut microbiota**. The gut microbial composition can be modulated by different interventions such as prebiotics, probiotics, FMT, and intermittent fasting, all of which are considering as potential therapeutics for T2D. Host genetics, epigenetics, and immunity also modulate gut microbiota. Some T2D medication improves circulating glucose levels partly through modulating gut microbiota, which further supports the usability of the gut microbiota as therapeutics for T2D.

Lactobacillus, *Bacteroides, Roseburia*, and *Akkermansia* can decrease pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-17, and tumor necrosis factor (TNF)-α (40, 50–52). Conversely, *Fusobacterium nucleatum* and *Ruminococcus gnavus* can increase inflammatory cytokine production. Therefore, depending on the composition, the gut microbiota may contribute to increased or decreased low-grade inflammation, impacting insulin sensitivity and T2D.

Gut microbiota profiling performed on large cohorts of T2D patients has found the abundance of several bacteria enriched or depleted in T2D subjects compared to controls (**Table 1**). In general, T2D patients commonly have a decreased abundance of SCFA producing bacteria (*Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Roseburia inulinivorans, Akkermansia*, and *Bifidobacterium*) and tryptophan metabolite producing bacteria (*Lactobacillus, Bacteroides, Bifidobacterium, Peptostreptococcus, Ruminococcus, Ruminiclostridium*, and *Clostridium*), and an increased abundance of opportunistic pathogens (*Bacteroides caccae* and *Clostridium hathewayi)*, branch chain amino acid synthesizing bacteria (*Bacteroides vulgatus* and *Prevotella copri*), and sulfate-metabolizing bacteria (*Desulfovibrio*, *Lactobacillus gasseri*, and *Lactobacillus reuteum*) compared to healthy controls (17–22, 26, 53). However, we note that not all the data derived from observational studies have been consistent. For example, one study comparing significant differences in gut microbiota diversity between T2D patients and healthy individuals was conducted on fecal samples from 18 men (24). In this study, decreased *Clostridia* and increased *Bacteroidetes* and *Proteobacteria* were observed, while overall diversity of the gut microbiota was positively correlated with plasma glucose levels in T2D patients. However, these results have not been identified in three large-scale metagenomics analyses performed in Europe and China (17, 18). A decrease in *Prevotella* was observed in 50 Japanese T2D patients compared to the healthy subjects (53), but in studies of 291 Nigerians and 171 Chinese, increased *Prevotella* abundance was associated with T2D (23, 25). The reason for the discrepancy between studies may be due to a number of confounding variables such as diet, genetics, medication use, and sequencing techniques. Utilizing alternative approaches and

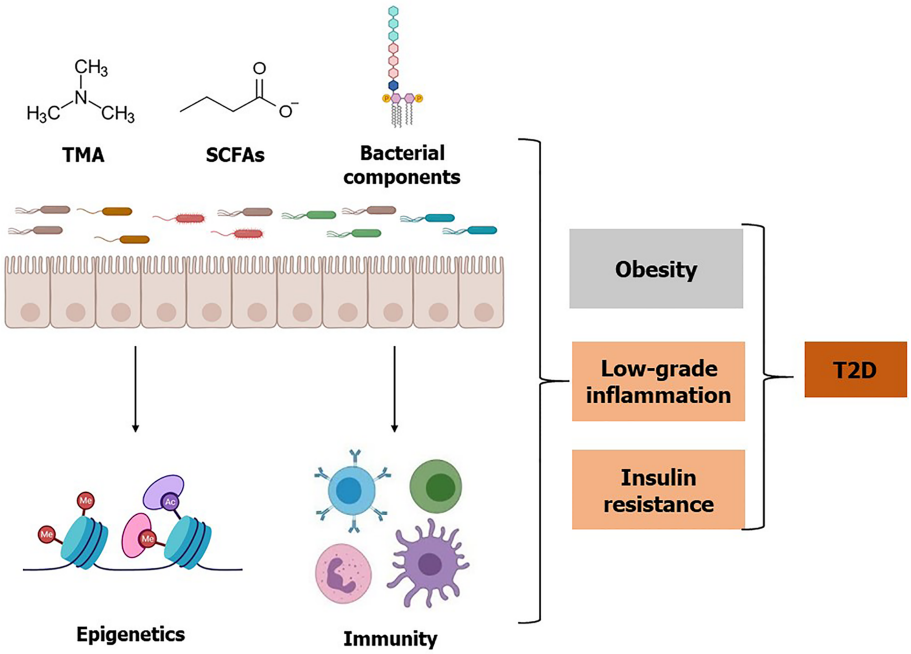

FIGURE 2 | **Effects of gut microbiota, microbial metabolites, and bacterial components on T2D**. Gut microbiota and specific bacterial taxa are associated with a risk of obesity, low-grade inflammation, and insulin resistance. Microbial metabolite TMA is converted to TMAO by the host enzyme and elevated TMAO is associated with insulin resistance. Whereas some bacterial metabolites such as SCFAs may improve glucose homeostasis. Additionally, SCFAs influence epigenetic programming by inhibiting histone deacetylase enzyme activity, which may improve insulin resistance and T2D. Besides live bacteria, bacterial components such as LPS, flagellin, and peptidoglycan can elicit an inflammatory response and may contribute to the increased risk of T2D. Conversely, some bacterial components such as Amuc 1100 can improve T2D. Referred studies can be found on the main body of this review.

developing new technologies are of critical need to determine which of the associations between the microbiota and T2D are causal and which of the microbial differences are responsive to T2D.

MICROBIAL METABOLITES

Beyond the direct effects of bacteria in the microbiota or their components, such as LPS, we now appreciate that the microbiota is a metabolically active "organ" that produces small biomolecules. In the following section, we highlight two important bacteria-derived metabolites TMAO and SFCAs, and briefly comment on other metabolites important for T2D. Gut microbiota is involved in the digestion of food ingredients and produces metabolites supporting physiological function in the human body (54). Microbial metabolites assist our interpretation of the underlying mechanisms by which gut bacterial taxa influence host health and disease (**Figure 2** and **Table 2**).

Trimethylamine N-Oxide

Trimethylamine (TMA) is produced by intestinal microbial metabolism of dietary choline and carnitine and is transported to the liver *via* the portal vein. In the liver, TMA is converted to TMAO by the activity of flavin-containing monooxygenase 3 (FMO3) (70, 71). *In vivo* studies have identified several candidate microbial taxa associated with TMA/TMAO production including *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *C. hathewayi*, *C. sporogenes*, *Desulfovibrio desulfuricans*, *Edwardsiella tarda*, *Escherichia fergusonii*, *Proteus penneri*, and *Providencia rettgeri* (55). TMAO concentrations are elevated in T2D patients, suggesting that this pathway is associated with T2D (72, 73). However, it is not yet clear if the elevated TMAO has a direct causal effect on T2D development or if it is a consequence of T2D. Animal studies have shown that TMAO consumption impairs glucose tolerance by mediating the insulin signaling pathway in the liver and upregulates the expression of pro-inflammatory mediators in adipose tissue (56). Reduction of plasma TMAO by FMO3 knockdown also decreases plasma glucose and insulin levels, whereas FMO3 overexpression increases plasma glucose level and induces insulin resistance (74). A similar relationship between TMAO and T2D may exist in humans as circulating TMAO concentration was found to be significantly higher in T2D patients compared to control subjects observed in a meta-analysis (75). In contrast, a recent Mendelian randomization analysis suggests that elevated circulating TMAO is a consequence of T2D not causal (76). Additional studies on the relationship between TMAO and T2D are needed to clarify these results.

Short-Chain Fatty Acids

SCFAs are microbial metabolites produced in the colon and are known to have a wide range of biochemical effects on the host (77).

TABLE 2 | Role of microbial metabolites on T2D.

Metabolites	Metabolite production pathway	Metabolite-producing bacteria (genus)	Mechanism on T2D risk	References
TMAO	Choline (diet) -> TMA (intestine) -> TMAO (liver)	• TMA: *Anaerococcus, Clostridium, Desulfovibrio, Edwardsiella, Proteus, Providencia*, and others	- Impair glucose tolerance by mediating the insulin signaling pathway in the liver - Increased HOMA-IR, exacerbated the impaired glucose tolerance, and upregulate expression of pro-inflammatory mediators in adipose tissue	Qi et al. (55) Gao et al. (56)
SCFA (Acetate, propionate, and butyrate)	Fiber (diet) -> Acetate, propionate, and butyrate (intestine)	• SCFA: *Anaerostipes, Blautia, Coprococcus, Eubacterium, Faecalibacterium, Marvinbryantia, Megasphaera, Roseburia, Ruminococcus*, and others	- Improve glucose metabolism and energy homeostasis - Increase intestinal glucose production and epithelial barrier function by promoting epithelial growth and *Bacteroides* species - Regulate the intestinal immune system by binding GPR41, GPR43, and GPR109A - Reduce plasma glucose level, appetite, insulin secretion, and slow gastric emptying by stimulating GLP-1 and GLP-2 secretion	Morrison et al. (57) Hirasawa et al. (58) Hamer et al. (59) Ross et al. (60)
Imidazole propionate	Histidine (diet) -> Imidazole propionate (intestine)	• Imidazole propionate: *Citrobacter, Dickeya, Eggerthella, Lactobacillus, Pectobacterium Staphylococcus*, and *Streptococcus*	-Suppress insulin signaling by mediating the activation of signaling pathways and insulin receptor substrates including rapamycin complex 1 (mTORC1)	Koh et al. (61)
Tryptophan metabolites (tryptamine, indole, indolelactic acid (ILA), indolepropionic acid (IPA), indoleacetic acid (IAA), and skatole	Tryptophan (diet) -> tryptamine, indole, ILA, IPA, IAA, and skatole (intestine)	• All tryptophan metabolites: *Clostridium* • Tryptamine: *Ruminococcus* • ILA: *Lactobacillus* and *Bifidobacterium* • IPA: *Peptostreptococcus* • ILA, IAA, and skatole: *Bacteroides*	- Reduce plasma glucose level, appetite, insulin secretion, and slow gastric emptying by stimulating GLP-1 secretion - Enhance the intestinal epithelial barrier by acting on the pregnane X receptor - Stimulate gastrointestinal motility by stimulating serotonin secretion - Activate the immune system by acting on the aryl hydrocarbon receptor - Exert anti-inflammatory and anti-oxidative effects in the systemic circulation	Roager et al. (62) Dodd et al. (63) Venkatesh et al. (64)
Bile acids (BA)	Cholesterol (liver) -> Primary BA (liver) -> Secondary BA (intestine)	• Secondary BA: *Bacteroides, Bifidobacterium*, *Clostridium, Eubacterium, Lactobacillus*, *Listeria, Peptostreptococcus*, and *Ruminococcus*	- Bind with host nuclear receptors such as FXR (Farnesoid X receptor), PXR (Pregnane X receptor), vitamin D Receptor, RAR-related orphan receptor gamma, and G-protein coupled membrane receptor (TGR-5) and modulate insulin sensitivity, and gluconeogenic genes expression	Jia et al. (65) Chiang et al. (66) Zhou et al. (67)
Branched-chain amino acids (BCAA)	Glucose, amino acid (diet) -> BCAA (intestine)	• BCAA: *Lactobacillus*, *Leuconostoc*, and *Weissella*	- Interfere with insulin signaling *via* phosphorylation of insulin receptor substrate-1 (IRS-1) on serine residue by stimulating rapamycin and its downstream effector, mTOR/S6 kinase	Chen et al. (69) Mutaguchi et al. (173)

Anaerostipes, *Blautia*, *Coprococcus*, *Eubacterium*, *Faecalibacterium*, *Marvinbryantia*, *Megasphaera*, *Roseburia*, and *Ruminococcus* are among the primary gut microbes that produce SCFAs. The SCFAs acetate and butyrate improve glucose homeostasis by inducing intestinal production of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). These peptides in turn stimulate insulin secretion, suppress appetite, and slow gastric emptying (78–80). GLP-1 is released from colonic enteroendocrine L cells that are distributed along the length of the intestinal epithelium and are in direct contact with the gut microbiota (81). Additionally, SCFAs can regulate the intestinal immune system through G protein-coupled receptors (GPCRs) such as GPR41 and GPR43 (82). Reduced abundance of SCFA producing bacteria was observed in T2D patients suggesting that this pathway is altered in T2D (17, 83). A clinical trial demonstrated that selective enrichment of SCFA producing bacteria, achieved by dietary fiber supplementation, was associated with lower hemoglobin A1c (HbA1c) levels and improved glucose metabolism (84). Stool samples collected prior to and following the intervention were then mechanistically tested *via* adoptive transfer experiments using mice to establish causality (84). Overall, SCFAs are involved in glucose and lipid metabolism *via* activation of SCFA receptors (85). Therefore, SCFA could be an intermediate phenotype by which microbiota provides a beneficial effect of T2D prevention.

Besides TMAO and SCFAs there are several other microbial metabolites such as tryptophan catabolites: tryptamine, indole, indolelactic acid (ILA), indolepropionic acid (IPA), indoleacetic acid (IAA), indoleacrylic acid (IA), indolealdehyde (IAld), and 3-methylindole (skatole) [reviewed in (62)]; bile acids: deoxycholic acid (DCA) and lithocholic acid (LCA) [reviewed at (65, 86)], histidine catabolite: imidazole propionate (Imp) (87), branched-chain amino acids [reviewed at (88)], and hydrogen sulfide (84) have been investigated in the context of T2D (89–92). These microbial metabolites are involved in the regulation of host metabolism, immunity, gene expression, and intestinal integrity, creating an important link between the gut microbiota, and insulin resistance and T2D development. Individually, these results are intriguing but cumulatively the results are complex and heterogeneous. Alterations in the experimental system, environment, diet, or even circadian rhythms (93) all have been implicated as sources of variation contributing to the heterogeneity in the literature. Of particular interest to this review, the overall variation in the microbiota composition was better captured by a 17-day diet history (94). Therefore, information from longitudinal sampling and metabolomics is required to identify precise and dynamic interactions between diet, microbiota, and host.

INTERACTION BETWEEN HOST GENETICS AND GUT MICROBIOTA ON T2D

In addition to environmental factors including gut microbiota and microbial metabolites, host genetic architecture is associated with T2D (13). Many studies demonstrate that host genetics influences the community structure of gut microbiota in humans. This opens several interesting hypotheses regarding host-microbe symbiosis and perhaps the microbiota as a mediating variable contributing to the missing heritability in GWAS. More specifically, the contribution of genetic polymorphisms associated with T2D may be partly mediated through gut microbiota. One could wonder if intervention in the gut microbiota may improve T2D in susceptible individuals. Here we provide some recent evidence implicating interactions between host genetics and the microbiome that affect T2D.

Human studies of monozygotic and dizygotic twins have demonstrated that host genetics contribute to the composition of the microbiota (12), by tolerating or rejecting several microbial taxa. For example, the abundance of *Bifidobacterium*, an important commensal bacterium for T2D, is associated with host genotype at the lactase gene locus (LCT, rs4988235, and rs1446585) (95). Individuals with the GG genotype have reduced lactase activity and harbor higher levels of *Bifidobacterium* in their gut. Mechanistically these individuals provide more lactose to the bifidobacteria for utilizing as an energy source, which enriches bifidobacteria in their gut. Establishing if an increase in *Bifidobacterium* due to LCT genotype affects T2D remains to be determined.

In addition to the LCT locus and *Bifidobacterium*, genetic studies are beginning to identify some bacteria associated with specific genetic loci. For example, a recent GWAS found an association between *Ruminococcus* and rs150018970 near the gene *RAPGEF1* (96). RAPGEF1 is a signaling protein that transduces signals from GPCRs, which are involved in the regulation of gastrointestinal tract physiology, such as metabolism, immune cell differentiation, and tissue repair (97). Similarly, another study (98) found a quantitative trait locus for *Butyricicoccus* at the locus of SLC5A11 (rs72770483), which encodes a sodium-dependent myo-inositol/glucose co-transporter protein (99). These studies underscore the influence of host genetics on gut microbial colonization. However, further studies will be needed to determine to what extent host genetics affects the gut microbiota and T2D.

In addition to interactions between the host genotype and microbiota composition, we are now beginning to appreciate that microbial metabolites can influence host gene expression through epigenetic mechanisms (15). Thus, there seem to be bi-directional interactions with effects on both the host and specific bacteria of the microbiome. For example, in various tissues, including proximal colon, liver and white adipose tissue, microbial metabolites such as SCFAs influence epigenetic programming by inhibiting histone deacetylase (HDAC) enzyme activity, (100), which promotes de-condensation and relaxation of chromatin and increases chromatin accessibility to transcription factors (101). In particular, *Faecalibacterium prausnitzii* is one of the most abundant anaerobic bacteria in the healthy human gut that produces butyrate. Butyrate targeted inhibition of HDAC1 may have anti-inflammatory effects and ultimately improve insulin sensitivity by downregulating the IL-6/STAT3/IL-17 pathway (102). Butyrate may also influence

differentiation of Th17 and T_{reg} cells through enhanced Forkhead Box P3 (*Foxp3*) expression (102). In human adipose tissue, the epigenetic regulation of the expression of genes involved in glucose and energy homeostasis, such as insulin-like growth factor 2 mRNA binding protein 2(*IGF2BP2*), is associated with gut bacterial populations (103). These data support the idea that the gut microbiota could act as an epigenetic regulator in T2D (104). A genome-wide DNA methylation analysis of isolated human pancreatic islet cells harvested from donors with and without T2D revealed 853 unique differential DNA methylation genes, including 17 genes previously identified in GWAS such as *TCF7L2*, *THADA*, *KCNQ1, FTO*, and *IRS1* associated with the risk of T2D (105). This reinforces the idea that genetic and epigenetic mechanisms may interact to affect pancreatic β-cell function, development of insulin resistance, and T2D. Understanding if the microbiota specifically aids the host epigenetic changes associated with T2D could be important in the development of novel therapies T2D or comorbidities such as obesity.

THERAPEUTIC POTENTIAL OF GUT MICROBIOTA FOR T2D

The associations between gut microbiota, microbial metabolites, and T2D, opened a new perspective for potential novel therapeutics for T2D. Several gut microbiota targeted therapeutics including fecal microbiota transplantation (FMT), medication, and dietary choices could be useful therapeutic strategies to manage T2D (**Figure 1**). Several clinical trials to evaluate the impact of these potential therapeutic agents on T2D are currently completed or in progress (**Table 3**).

Fecal Microbiota Transplantation

FMT has gained attention over the past few years as a research method demonstrating the contribution of gut microbiota to a disease state. Most clinical trials with FMT have been performed in patients with *Clostridium difficile* infections (106, 107) and these studies have been successful. As an extension of these studies, several additional diseases such as T2D have been suggested responsive to microbiota transplantation (108). In rodent models, insulin sensitivity significantly improved after transferring microbiota in MyD88 deficient NOD mice (109). Similar studies where human microbiota from healthy Chinese subjects are transplanted into diabetic db/db mice remarkably lowers fasting blood glucose concentrations (110). Likewise, transplantation of fecal samples of patients treated with metformin into germ-free mice improves glucose tolerance (111). A limited number of studies have begun to suggest that FMT from lean subjects into patients improves insulin sensitivity which could be in part due to increased butyrate-producing bacteria (108). One study examined the effects of lean donor versus self-FMT on metabolic syndrome patients and found that insulin sensitivity improves significantly at 6-weeks after FMT in male recipients with the metabolic syndrome (112). However, FMT treatment sometimes failed to improve targeted clinical phenotypes. For example, one study failed to show reduced TMAO levels in the recipient of FMT from a vegan donor (112), who have altered intestinal microbiota compared to omnivores (114) and low production of TMAO (115). In addition to inconsistent results, the long-term effects of FMT have not been adequately examined. Thus, further studies are needed to evaluate the long-term effectiveness and potential side-effect of FMT in humans.

Anti-Diabetic Drugs

Metformin is a widely known common treatment for T2D but the exact mechanisms underlying the hypoglycemic effect are not yet fully understood. Metformin has been shown to have an inhibitory effect on T2D by activating AMP-activated protein kinase (AMPK) or inhibiting mitochondrial respiration and glycerophosphate dehydrogenase (116–118). Recently, evidence has been reported suggesting that the composition of the gut microbiota mediates the efficacy of metformin to lower blood glucose levels. The fact that intravenous injection of metformin, unlike oral administration, does not lower hyperglycemia, suggests that gut microbiota is an important part of metformin action (119). Indeed, metformin shifts the composition of gut microbiota in both mice and humans, making them more similar to the microbiota of a healthy host (111, 120, 121). Some of these gut microbiota changes have also been seen in healthy people who have not responded to glycemic control to metformin treatment, thus suggesting shifts in the gut microbiota induced by metformin itself, rather than simply reflecting lowered blood glucose level. Metformin influences the abundance of several microbial taxa, including increased abundance of *A. muciniphila*, *Bifidobacterium bifidum*, *Bilophila wadsworthia*, *Escherichia*, *Lactobacillus, Shigella* spp. as well as a reduced abundance of *Clostridium* spp. and *Intestinibacter* spp. (111, 122, 123). Regarding the effects of these changes on blood glucose, metagenomic analysis of microbial composition demonstrates changes in various functional pathways affecting the production of propionate and butyrate (124, 125). Metformin stimulates the activity of endocrine cells by regulating bile acid conversion, improving intestinal permeability, reducing endotoxin levels, and enhancing the release of GLP-1 and PYY peptides (126). Metformin also decreases the TMA level and the growth of bacteria that produce it in the gut, and thus the circulating TMAO level in mice (127). The fact that transferring the microbiota from metformin-treated mice improves metabolic traits in aged mice indicates that the shifts in the gut microbiota by metformin treatment are beneficial (111). The effect of the microbiota on the efficacy of metformin remains unclear as a recent study found that metformin's ability to improve T2D in mice was not affected by the elimination of gut microbiota using gnotobiotic mice or antibiotics (128). Although previous studies did not directly demonstrate the role of gut microbiota in improving glycemic control by metformin, it is suggested that the anti-inflammatory activity of metformin could potentially play a role in eliciting some beneficial effects regardless of the gut microbiota.

Another anti-diabetic drug with a link to the microbiota is Acarbose, an α-glucosidase inhibitor. Acarbose suppresses the

TABLE 3 | Ongoing or completed clinical trials on T2D with FMT, medication, prebiotics/functional foods, or probiotics.

Category	NCT Number	Title	Interventions	Country	Age	Phases	Enrollment
FMT	NCT02346669	Fecal Microbiota Transplantation for Diabetes Mellitus Type II in Obese Patients	FMT	Israel	18–65	Phase 2	30
	NCT01790711	Fecal Microbiota Transplantation on Type 2 Diabetes Mellitus	FMT	China	18–70	Phase 2| Phase 3	30
	NCT03127696	Randomized Placebo-controlled Study of FMT to Impact Body Weight and Glycemic Control in Obese Subjects With T2DM	FMT	China	18–70	NA	61
Medication	NCT03018444	The Effect of HMG-CoA Reductase Inhibition on Postprandial GLP-1 Secretion	Atorvastatin	Denmark	18–70	NA	15
	NCT02900417	Evaluation of the Effect of Sitagliptin on Gut Microbiota in Patients With Newly Diagnosed Type 2 Diabetes	Sitagliptin	China	40–70	NA	9
	NCT02061124	Effect of Bile Acid Sequestration on Postprandial GLP-1 Secretion, Glucose Homeostasis and Gut Microbiota	Sevelamer 1600 mg for 7 days	Denmark	35–80	NA	50
	NCT02960659	Therapeutic Targets in African-American Youth With Type 2 Diabetes	Metformin and Liraglutide	USA	12–25	Phase 1	92
	NCT04426422	Effect of Metformin on Gut Microbiota Changes and Glycemic Control of Newly Diagnosed Type 2 Diabetes	Metformin Hydrochloride	China	18–65	Phase 4	52
	NCT01758471	Efficacy of Acarbose on Intestinal Microbiome and Incretins of Type 2 Diabetes	Glipizide | Acarbose	China	40–60	Phase 4	160
	NCT04057261	Effect of Liraglutide on the Metabolic Profile in Patients With Type 2 Diabetes and Cardiovascular Disease	Liraglutide	Germany	18–	Phase 3	50
	NCT02583438	Evaluate the Effect of Saxagliptin on Gut Microbiota in Patients With Newly Diagnosed Type 2 Diabetes	Saxagliptin	China	20–65	Phase 4	100
	NCT04287387	Response of Gut Microbiota in Type 2 Diabetes to Hypoglycemic Agents	Glucophage | Acarbose | Sitagliptin | Dapagliflozin | Pioglitazone | Glimepiride Tablets	China	18–65	Phase 4	180
Prebiotics/ Functional foods	NCT03557541	Sardine-enriched Diet for Prevention Type 2 Diabetes	Sardine diet	Spain	65–	NA	182
	NCT03708887	The Effect of Omega-3 FA on Glucose and Lipid Homeostasis Disorders in Obese/Diabetic Patients	Omega-3 fatty acid		50–70	Phase 4	900
	NCT03194152	Peanut Consumption and Cardiovascular Disease Risk in a Chinese Population	Peanut	USA	20–65	NA	238
	NCT04403217	Effect of MEDiterranean Diet on the microBIOME of Individuals With Type 2 Diabetes	Individualized structured dietary plan	Portugal	40–80	NA	30
	NCT02294526	A Sardine Diet Intervention Study to Assess Benefits to the Metabolic Profile in Type 2 Diabetes Mellitus Patients	Sardine diet	Spain	40–85	NA	35
	NCT02717078	The LoBAG Diet and Type 2 Diabetes Mellitus	Diet Therapy	USA	18–	NA	50
	NCT03120299	The Effect of Omega-3 FA on Hypertriglyceridemia in Patients With T2DM(OCEAN)	Omega-3 fatty acid	China	20–75	Phase 4	350
	NCT02929901	The Effects of Coffee Main Constituents (Caffeine and Chlorogenic Acid) Supplementation on Inflammatory, Metabolic Factors, Hepatic Steatosis and Fibrosis in None- Alcoholic Fatty Liver Patients With Type 2 Diabetes	Caffeine and chlorogenic acid	Iran	30–65	Phase 2| Phase 3	200
	NCT03141710	Commercial Prebiotic Supplement Study	Prebiotics	Scotland	18–65	NA	12
	NCT03552991	Effects of Dietary Fiber on Glucose Control in Subjects With Type 2 Diabetes Mellitus	Agiocur Pregranules	South Korea	50–	Phase 4	14
	NCT02974699	Role of Gastrointestinal Microbes on Digestion of Resistant Starch and Tryptophan Availability to Humans	Potato Starch | Pregelatinized Starch	USA	18–65	Early Phase 1	20
Probiotics	NCT01765517	Study to Explore the Effects of Probiotics on Endotoxin Levels in Type 2 Diabetes Mellitus Patients	Probiotics	Saudi Arabia	20–75	NA	83
	NCT02728414	Probiotics Effect on Glucose and Lipid Metabolism and Gut Microbiota in Patients With Type 2 Diabetes	Probiotics	China	20–80	NA	100
	NCT04089280	Probiotics in Metformin Intolerant Patients With Type 2 Diabetes	Sanprobi Barrier-multispecies probiotics	Poland	18–75	NA	50
	NCT03037918	Effect of Yakult Ingestion on Diet-induced Insulin Resistance in Humans	Yakult light	England	18–30	NA	56
	NCT01250106	Probiotics as a Novel Approach to Modulate Gut Hormone Secretion and Risk Factors of Type 2 Diabetes and Complications	*Lactobacillus reuteri*	Germany	40–65	Phase 1| Phase 2	20
	NCT04495972	Intestinimonas for Prevention of Type 2 Diabetes Mellitus	*Intestinimonas*-capsules	Netherlands	18–65	Early Phase 1	26

(Continued)

TABLE 3 | Continued

Category	NCT Number	Title	Interventions	Country	Age	Phases	Enrollment
	NCT01836796	Metabolic Effects of Lactobacillus Reuteri DSM 17938 in Type 2 Diabetes	*Lactobacillus reuteri*	Sweden	50–75	NA	46
	NCT04296825	Effect of Camel Milk With Probiotic on Type 2 Diabetes Mellitus	Camel milk containing *Bifidobacterium animalis A6* | Camel milk | *Bifidobacterium animalis A6* | Cow milk	China	35–68	Phase 1	45
	NCT02861261	A Study on the Efficacy and Gut Microbiota of Berberine and Probiotics in Patients With Newly Diagnosed Type 2 Diabetes	Berberine hydrochloride tablets and ProMetS probiotics powder	China	20–69	Phase 3	400
	NCT00699426	The Effect of Nexium and Probiotics on Insulin Secretion and Cardiovascular Risk Factors in Patients With Type 2 Diabetes	Nexium | Yoghurt	Denmark	40–70	Phase 3	41
	NCT03377946	Effect of Probiotics on Pre-diabetes and Diabetes in China	Probiotics	China	18–60	NA	220
	NCT01752803	RCT Examining Effects of Probiotics in T2DM Individuals	Probiotics	Malaysia	30–65	NA	100
	NCT01620125	Metabolic Control Before and After Supplementation With Lactobacillus Reuteri DSM 17938 in Type 2 Diabetes Patients	*Lactobacillus reuteri*	Sweden	50–80	Early Phase 1	12

T2D, type 2 diabetes; NAFLD, nonalcoholic fatty liver disease; FMT, fecal microbiota transplantation; HbA1C, hemoglobin A1C; IR, insulin resistance; HOMA, homeostasis model assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TNF-α, tumor necrosis factor-alpha; IL, interleukin; CRP, C-reactive protein; OGTT, oral glucose tolerance test. (Data from https://clinicaltrials.gov).

conversion of oligosaccharides to monosaccharides and disaccharides, delays the absorption of glucose in the intestine, and lowers blood glucose levels after a meal. Due to its effects on carbohydrate metabolism, Acarbose has been hypothesized to affect microbiota composition. In T2D patients Acarbose treatment alters the gut microbiota. The abundance of *Dialister, B. longum*, *Faecalibacterium*, and *Lactobacillus* increases, while the abundance of *Butyricoccus*, *Phascolarctobacterium*, and *Ruminococcus* is reduced. These changes in composition may improve gut health as evidenced by the decrease in circulating LPS levels (129–131). This alteration in the gut microbiota composition after Acarbose treatment suggests that the therapeutic effect of Acarbose may be partially mediated through microbiota. Whether these changes in microbial composition contribute to acarbose's effect on lowering blood glucose has not been extensively studied. Similarly, liraglutide, a GLP-1 receptor agonist, stimulates satiety, slows gastric emptying, inhibits glucagon, and promotes insulin secretion. In animal studies, liraglutide increased the abundance of *A. muciniphila, Allobaculum*, *Anaerostipes*, *Blautia*, *Butyricimonas*, *Desulfovibrio*, *Lactobacillus*, *Turicibacter*, and SCFAs producing bacteria and decreased the abundance of Bacteroidales, Clostridiales, Proteobacteria (132, 133). These data suggest that the beneficial effect on hyperglycemia these drugs have, may in part be through the gut microbiota, although further clinical studies are needed.

Probiotics

Probiotics are live microorganisms that have a beneficial effect on human health (134). Various beneficial effects of taking probiotics have been reported, including improving gut health, alleviating symptoms of lactose intolerance, inhibiting the growth of pathogenic bacteria, producing SCFAs, balancing pH, and stimulating the immune system (135). The use of probiotics to manage T2D is of interest, but a limited number of studies have evaluated the effects in clinical settings. Preliminary studies indicated that alteration of the gut microbial composition by probiotics supplementation might improve T2D by reducing pro-inflammatory cytokines, intestinal permeability, and oxidative stress [reviewed at (136)]. Several bacterial species are used in commercial probiotics supplement products, including *Bifidobacterium longum* subsp. *infantis, Lactobacillus, Streptococcus, Pediococcus*, and *Lactococcus species* (137). *L. gasseri*, *Lactobacillus helveticus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* probiotic reduce fasting blood glucose levels with HbA1c (138–140). Mechanistically, these probiotics have been shown to have antioxidant and immunomodulatory effects by reducing oxidative stress (140), reducing inflammatory molecules, and inhibiting effector functions of $CD4^+$ T-cells (142), which may influence on the reducing blood glucose levels and T2D risk. A randomized, double-blind, placebo-controlled trial of administration of *A. muciniphila* in overweight/obesity insulin-resistant volunteers improved insulin sensitivity and reduced insulinemia, plasma total cholesterol, body fat mass, hip circumference, and level of blood markers associated with liver dysfunction and inflammation (32). Recent meta-analysis studies showed that the probiotic supplementation improved the fasting blood glucose, HbA1c, and homeostatic model assessment for

insulin resistance (HOMA-IR) in T2D patients and thus can be recommended as complementary advice alongside medicine and lifestyle modifications for T2D treatment (143, 144).

Prebiotics

Prebiotics are the non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and (or) the activity of one or a limited number of bacterial species already resident in the colon (145). Inulin, a linear β-2,1 fructosyl-fructose polydisperse carbohydrate material with or without a α-D-glucose moiety (146), is one of the most studied prebiotics. Inulin-type fructooligosaccharide (ITF) improved glycemia by increasing the production and release of the active forms of GLP-1 from the cecum and proximal colon and reducing plasma ghrelin concentration in the rat (147). The direct effect of inulin supplementation on the T2D is not conclusive in human clinical trials. One study reported that dietary inulin reduced fasting blood glucose, body weights, glycated hemoglobin, plasma LPS, IL-6, TNF-α and IL-17A in T2D patients (148). A recent placebo-controlled crossover clinical trial (149) found enrichment of *Bifidobacterium* and *Bacteroides* with a significantly higher fecal SCFAs concentration due to ITF consumption compared to placebo (150). Additionally, the relative abundance of *Cyanobacteria* and *Bacteroides* is increased, and a reduction in the relative abundance of *Ruminiclostridium*, *Deferribacteres*, and *Tenericutes* is observed due to inulin supplementation, indicating that the dietary inulin alleviates T2D *via* suppressing inflammation and modulating gut microbiota (148). A recent systemic review (151) has summarized clinical trials conducted to evaluate the effect of dietary inulin on *Akkermansia muciniphila*, which are usually present at a higher abundance in healthy individuals compared to T2D patients and found an increased abundance in the treatment group compared to controls. However, others found no effect largely due to interindividual variation at the baseline T2D phenotypes (152).

It should be noted that a symbiotic mixture of prebiotics and probiotics (134), supplementation could provide a better beneficial effect compared to prebiotic or probiotic alone (153). For example, *Lactobacillus acidophilus* DSM20079 induces 14·5-times more butyrate in the presence of inulin or pectin than glucose (154). Berberine, a natural plant alkaloid extracted from *Berberis aristata* and *Coptis chinensis*, is reported to be an effective remedy for T2D (155). A recent randomized, double-blind, placebo-controlled trial conducted in China demonstrates that administration of berberine with probiotics improves HbA1C levels compared to the group treated with berberine alone (156). A meta-analysis of randomized controlled trials reported that diets supplemented with either prebiotics or symbiotics improved fasting blood glucose and HbA1C in patients with T2D (157). Therefore, symbiotic products that selectively stimulate and (or) activate metabolism of probiotics could be recommended to effectively lower the risk of T2D.

Intermittent-Fasting

Intermittent fasting (IF) is defined as a periodic dietary restriction, which has been shown to increase lifespan, and to reduce the risk of developing various age-related pathologies including T2D (158). Animal studies of IF have reported an improvement in body composition, glucose and lipid metabolism, decreased inflammation, and autophagy (159) and gut microbiota might play a pivotal role in this process (160, 161). Though most of the human IF studies show a beneficial effect, the results are not completely conclusive. Two recent reviews summarize the recent literature on the effect of IF on T2D (162, 163). In this portion of the review, we will critically evaluate the microbial aspect of the IF on T2D. A recent study (164) using diabetic mice reported that a 28-day IF intervention re-structured the gut microbiota by increasing the abundance of *Aerococcus, Corynebacterium, Odoribacter*, and *Lactobacillus* and decreasing the abundance of *Streptococcus*, *Rummeliibacillus*, and *Candidatusarthromitu*, which reduced plasma glucose and insulin levels, and improved energy metabolism. The changes in bacterial abundances due to IF are correlated with plasma secondary BAs concentration, increased villi length and reduced gut leakage accompanied by decreased plasma LPS levels (164), indicating improved low-grade inflammation (165). More importantly, the effect of IF on the T2D was suppressed by antibiotics treatment (164), suggesting that the microbiota is a causative agent of improvement in T2D by IF. An alternative to IF is a fasting-mimicking diet (FMD), which contains very low calories and low protein (166). Intermittent administration of FMD led to the reconstruction of gut microbiota by increasing the genera of *Parabacteroides* and *Blautia* while reducing *Prevotellaceae, Alistipes*, and *Ruminococcaceae*, along with normalized blood glucose levels, improved insulin sensitivity and β cell function in hyperglycemic db/db mice. This study further underscores that the loss of pancreatic islets and β cells can be prevented by the FMD-mediated altered gut microbiota (167), indicating that FMD improved T2D through pancreatic β cells function. Overall, IF may modulate gut microbiota and improve T2D. However, these findings need to be validated in human cohorts using longitudinal studies to establish the long-term effectiveness of IF in health outcomes including T2D.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Substantial evidence suggests the gut microbiota, and the metabolites it produces, are critical to the etiology of T2D. A strategy including FMT, medication, prebiotics, probiotics, functional food, and intermittent fasting has been suggested as strategies to reduce T2D. However, most studies have focused on the characterization of gut microbiota rather than functional validation of specific microbial taxa affecting T2D risk. Identifying specific causally related microbial taxa or microbial metabolites responsible for the pathogenesis of T2D could provide interesting new opportunities for the diagnosis, treatment, and prevention of T2D.

Recently several novel approaches have been taken to directly modify the gut microbiota. For example, one study (168) reported a novel approach to remodeling the gut microbiota using cyclic d,l-α-peptides. Alternatively, the FXR agonist

fexaramine, which was not absorbed by the intestine, binds the FXR receptor on intestinal cells and induces enteric fibroblast growth factor 15 that leads to alterations in bile acid composition, reduces diet-induced weight gain, body-wide inflammation, and hepatic glucose production (169). These studies suggest that the development of therapeutics targeting the microbiome instead of the host is a viable strategy for T2D.

As discussed above, probiotic supplementation and FMT studies have established a causal relationship between gut microbiota and T2D. However, studies (170, 171) using FMT have demonstrated that the relationship between gut microbiota and disease phenotype is more complex than usual thought. For example, FMT may not always be able to transfer the beneficial clinical phenotype, instead sometimes can be resulted in a detrimental opposite effect. A recent FMT study (31) in which the gut microbiota of C57BL/6J mice ablated using antibiotics was reconstituted with either C57BL/6J or WSB/EiJ fecal microbiota. C57BL/6J mice are more susceptible to obesity, diabetes, and atherosclerosis compared to WSB/EiJ mice (170). Paradoxically, mice reconstituted with WSB/EiJ microbiota had significantly higher fat mass compared to the mice reconstituted with C57BL/6J microbiota. Moreover, among the members of gut microbiota, only the bacterial community is being studied extensively. To date, enteric virus, fungal, or archaea communities are still underappreciated mostly because of the assay difficulties and lack of standard reference databases and thus their contribution to T2D remains largely unknown. Therefore, incorporating these members in analysis may potentially lead to the development of novel therapeutics for T2D.

Computational approaches such as machine learning facilitate the analysis of large "-omics" datasets through the development of algorithms and mathematical models designed to predict outcomes. It remains to be determined how these novel computational approaches can be harnessed to further our understanding of the microbiota's role in T2D but initial studies are promising. Recently two studies used machine learning tools to explore the role of the microbiome in precision nutrition (172) and to predict cirrhosis based on gut-microbiota features (173). Thus, use of these novel computational approaches may further our understanding of the metabolic consequences of how alterations in dietary habits, microbiota, metabolomics, genetics, and epigenetics, interact to alter metabolism. A better understanding of the interactions between microbiota, lifestyle, and host factors such as genetics and epigenetics might lead to a novel therapeutic approach for T2D.

AUTHOR CONTRIBUTIONS

BB supervised all portions of the review process, interpreted the results, and mentored manuscript writing. MH and MK conducted the literature search, extracting the information, and drafting the manuscript. MH and MK also addressed co-authors' comments and concerns. BB, MH, and MK critically revised the manuscript. BB had primary responsibility for the final content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Roles of Gut Microbial Metabolites in Diabetic Kidney Disease

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Diabetes is a highly prevalent metabolic disease that has emerged as a global challenge due to its increasing prevalence and lack of sustainable treatment. Diabetic kidney disease (DKD), which is one of the most frequent and severe microvascular complications of diabetes, is difficult to treat with contemporary glucose-lowering medications. The gut microbiota plays an important role in human health and disease, and its metabolites have both beneficial and harmful effects on vital physiological processes. In this review, we summarize the current findings regarding the role of gut microbial metabolites in the development and progression of DKD, which will help us better understand the possible mechanisms of DKD and explore potential therapeutic approaches for DKD.

Keywords: gut microbiota, microbial metabolites, diabetic kidney disease, diabetes, short-chain fatty acids, Trimethylamine-N-oxide

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INTRODUCTION

With the continuous improvement of people's living standards, changes in lifestyle, and environmental factors, the incidence of diabetes mellitus (DM), which is a metabolic disorder, has been increasing year by year. According to data released by the International Diabetes Federation (IDF), about 463 million people worldwide were living with DM in 2019. The global prevalence of DM is expected to rise to rise to 10.2% (578 million people) by 2030 and to 10.9% (700 million people) by 2045 (1). Patients with DM are more prone to serious complications that contribute to increased mortality and reduced quality of life. About 30-40% of patients with DM develop diabetic kidney disease (DKD), which is one of the major complications of DM, and most cases progress to end-stage renal disease (2).

The hyperglycemic condition initiates multiple events that damage the kidney structurally and functionally, such as glomerular hyperfiltration, proteinuria, thickening of the glomerular basement membrane, mesangial matrix accumulation, podocyte damage and glomerulosclerosis (3). Renal hemodynamics changes, the renin-angiotensin-aldosterone system, oxidative stress, inflammatory responses and fibrosis are also major factors in the pathogenesis of DKD (4). Current therapy for DKD includes antihypertensive and antiproteinuric means, as well as the use of angiotensin receptor blockers and angiotensin converting enzyme inhibitors. In addition, sodium glucose cotransporter 2 (SGLT-2) inhibitors and glucagon-like peptide 1 (GLP-1) receptor agonists are novel diabetes medications that prevent kidney failure (5). However, these means have limited efficacy in preventing the progression of

DKD (6). The complicated pathogenesis of DKD has not yet been elucidated. Understanding the pathophysiology of DKD is crucial for its prevention and treatment.

Recent advances in high-throughput metagenomic sequencing technologies have increased our knowledge of the symbiotic relationship between the gut microbiota and its host (7). The gut microbiota, as an important environmental factor, has emerged as a crucial regulator of human health and disease (8–10). The metabolites produced by the gut microbiota may also have pathogenic or beneficial effects on the host. These metabolites and their end products may play key roles in the host's metabolic network (11), immune processes (12) and neurobiological processes (13).

Recently, metabolomic studies have found that the microbial metabolite profile is altered in patients with type 2 diabetes (T2D) (14) and DKD (15). Mounting evidence supports the critical role of the gut microbiota as a factor (either beneficial or harmful) in the development of T2D (11) and DKD (16). Moreover, a number of metabolites are produced by the gut microbiota, using dietary nutrients as precursors, suggesting that diet has an important impact on gut microbial metabolites. In this review, we offer a summary of short-chain fatty acids (SCFAs), trimethylamine-N-oxide (TMAO), bile acids (BAs), polyphenols, tryptophan-derived metabolites, branched-chain amino acids (BCAAs) and other metabolites that play important roles in the pathogenesis and progression of DKD. We also discuss the potential mechanisms of microbial metabolites and DKD.

Short-Chain Fatty Acids

SCFAs, including acetate, propionate, and butyrate, are produced by the microbial community through the fermentation of non-digestible carbohydrates. Acetate and propionate are mainly produced by Bacteroidetes, whereas butyrate is primarily generated by Firmicutes (17). SCFAs can be used by the host for the biosynthesis of lipids, cholesterol, and proteins or as an energy source by gut mucosal cells (18). The effects of SCFAs are in part mediated by G-protein coupled receptors (GPR41, GPR43, and GPR109A) and histone deacetylase (HDAC), which are related to oxidative stress, immune, and inflammatory responses (19–22).

SCFAs have been reported to have multiple beneficial regulatory roles in both type 1 diabetes (T1D) (23, 24) and T2D (25). Several studies have also indicated their important roles in DKD (**Table 1**). SCFAs, especially acetate and butyrate, inhibit oxidative stress and inflammation in mouse glomerular mesangial cells that have been induced by high glucose and lipopolysaccharides (26). Treatment with a high-fiber diet or directly treatment with SCFAs can both protect against the development of DKD in mice by regulating the key pathways and genes involved in innate immunity, inflammation, and macrophage recruitment. GPR43 and GPR109A are critical to SCFAs-mediated protection against DKD (31). Exogenous sodium butyrate administration can improve DKD by reducing inflammation and oxidative stress, and by ameliorating fibrosis, apoptosis, and DNA damage, and this has been proven in different animal models (19, 22, 31). Exogenous sodium butyrate can also could protect human glomerular mesangial cells against high glucose-induced pyroptosis (32). These studies suggest that SCFAs, especially butyrate, may act as potential therapeutic targets for DKD.

Although much evidence suggests that increased butyrate production benefits the host through antidiabetic effects, some

TABLE 1 | Beneficial and Harmful effect of SCFAs on DKD *in vivo* and *vitro*.

Supplement	Animal/Cell Type	Mechanism	References
SCFAs (acetate, butyrate, propionate)	Mouse glomerular mesangial cells (SV40-MES-13)	(+) GPR 43; oxidative stress (ROS↓, MDA↓, SOD↑) ↓; inflammation (ICAM-1↓, MCP-1↓, IL-1β↓) ↓	(26)
High-fiber diet, SCFAs (acetate, butyrate, propionate)	C57BL/6, Gpr43-/- and Gpr109A-/- mice; Mouse kidney tubular epithelial cells and podocytes;	(+) GPR43 and GPR109A; IL-6↓, IFNγ↓, CCL2↓, CXCL10↓; fibronectin↓, TGFβ↓	(23)
SCFAs (acetate, butyrate, propionate)	C57BL/6 mice; Mouse glomerular mesangial cells (SV40-MES-13)	(+) GPR43-β-arrestin-2 signaling; oxidative stress (ROS↓) ↓; NF-κB inflammatory signaling↓	(27)
NaB	SD rats	(-) HDACs; eNOS↓, iNOS↓; α-SMA↓, collagen I↓, fibronectin↓, TGF-β_1↓; NF-κB↓; apoptosis↓; DNA damage↓	(22)
NaB	C57BL/6 and Nrf2-/- mice	(-) HDACs; (+) NRF2	(28)
NaB	Human renal glomerular endothelial cells	(-) caspase 1-GSDMD canonical pyroptosis pathway; (-) NF-κB/IκB-α signaling pathway	(29)
NaB	db/db and db/m mice; Mouse mesangial cells (SV40-MES-13)	(-) micro7a-5p/P311/TGF-β_1 pathway	(30)
NaB	Normal rat kidney tubular epithelial (NRK–52E) cells	(-) HDAC2; oxidative stress (ROS↓, SOD↑, LDH↓) ↓	(20)

(+), active; (-), inhibit; SCFA, short–chain fatty acids; NaB, sodium butyrate; GPR 43, G-protein-coupled receptor 43; HDAC, histone deacetylase; NRF2, nuclear factor erythroid 2-related factor 2; GSDMD, gasdermin D; ROS, reactive oxygen species; MDA, Malondialdehyde; SOD, superoxide dismutase; ICAM-1, intercellular cell adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; IL-1β, interleukin-1 β; IL-6, interleukin-6; TGFβ, transforming growth factor-β; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; α-SMA, α-smooth muscle actin; LDH, lactate dehydrogenase.

studies have suggested that acetate may exacerbate DKD. In some animal models, plasma acetate levels have been reported to be positively correlated with the intrarenal angiotensin II protein, which has long been considered to be one of the initiators of DKD. Acetate might also be involved in the kidney injury of early DKD (33). In addition, acetate has been reported to dysregulate cholesterol homeostasis through activating GPR43, thereby contributing to the tubulointerstitial injury of DKD (34). Whether SCFAs production is beneficial or harmful when it comes to DKD remains to be further studied.

Trimethylamine-N-Oxide

Dietary choline, phosphatidylcholine, and L-carnitine are metabolized into trimethylamine (TMA) by intestinal commensal bacteria. A choline-utilization gene cluster (Cut) responsible for anaerobic conversion of choline to TMA was identified in the sulfate-reducing bacterium *Desulfovibrio desulfuricans*. *CutC and CutD*, which are crucial genes in the cluster, encoding for choline TMA-lyase and its activating protein. Moreover, in some bacteria of the gender *Acinetobacter and Serratia*, *CntA and CntB* gens encode the two subunits of the oxidoreductase enzyme necessary to convert L-carnitine into TMA (35). On the other hand, a *YeaW/YeaX* gene pair encodes some oxygenase and oxidoreductase enzymes with substrate promiscuity for betaine, γ-butyrobetaine, choline and L-carnitine. These genes, not only *CntA/CntB* and *YeaW/YeaX*, but also orthologs and homologs of them, can be found in a wide range of gut microbiota: Actinobacteria, Betaproteobacteria (*Achromobacter*), Firmicutes (*Sporosarcina*), and Gammaproteobacteria (*Citrobacter, E.coli, Klebsiella pneumoniae, Providencia, and Shigella*) (36).

Once TMA is produced, most TMA is oxidized into TMAO in the liver predominantly by the enzyme flavin-containing monooxygenase 3 (37). Some clinical studies have investigated the role of TMAO in predicting the prognostic outcomes and mortality of diverse diseases (38–40). In addition to its predictive value, TMAO has been implicated in the pathogenesis of various human diseases, including cardiovascular (41), kidney (42), metabolic (43) and neurological (44) disorders.

TMAO is linked to the pathogenesis of many diseases by activating inflammatory pathways, such as nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome signals (45–48) and nuclear factor-κB signals (48–50), resulting in the release of inflammatory cytokines. Moreover, some studies have indicated that TMAO and its precursor can contribute to the pathogenesis of cardiovascular diseases by inducing endothelial dysfunction (51–54). In addition, both inflammation and endothelial dysfunction play important roles in the pathogenesis of DKD.

Recently, it has been widely accepted that increased levels of circulating TMAO directly contribute to renal dysfunction by promoting inflammation, oxidative stress, and fibrosis. Both TMAO-supplemented and choline-supplemented mice have shown elevated TMAO levels, which were associated with increases in tubulointerstitial fibrosis and collagen deposition (42). In a mouse model of high-fat-diet-induced obesity, elevated TMAO levels were found to promote renal oxidative stress and inflammation, subsequently contributing to renal interstitial fibrosis and dysfunction (55). In rats with chronic kidney disease (CKD), elevated TMAO levels promote vascular oxidative stress and inflammation, contributing to endothelial dysfunction (56). In a CKD mouse model, dietary supplementation with either choline or TMAO was found to significantly augment multiple indices of renal functional impairment and fibrosis (57). Supplementation with 3,3-dimethyl-1-butanol (an inhibitor of trimethylamine formation) or iodomethylcholine (an inhibitor of prototypic mechanism-based gut microbial choline TMA-lyase) can reduce plasma TMAO levels and prevent adverse renal structural and functional alterations in animal models (55–57). Therefore, high TMAO levels may exacerbate DKD, and TMAO inhibitors may have therapeutic potential to ameliorate DKD (**Figure 1**).

The regulation of TMAO in the DKD still warrants more investigation. Despite growing interest in TMAO biology, the receptor for TMAO is not yet known. Recent evidence has shown that TMAO directly bounds to and activates the protein kinase R-like endoplasmic reticulum kinase, which is an endoplasmic reticulum stress kinase (43). The next milestone is to identify the direct targets of TMAO.

Bile Acids

BAs are host-microbial co-metabolites and important signaling molecules. The primary BAs produced by the host can be metabolized by the gut microbiota into a secondary BAs (58). When a small portion of unabsorbed BAs enter the distal ileum, cecum, and colon, they undergo various reactions *via* microbiota: deconjugation, dehydroxylation, oxidation and epimerization reactions (59, 60). BA deconjugation is driven by bile salt hydrolase, which have been identified in *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, and *Lactobacillus* (61). Dehydroxylation occurs after deconjugation, and is catalyzed by members of the Firmicutes phylum, including Clostridium (*C. scindens* or *C. hylemonae*) and *Eubacterium*. Oxidation and epimerization require BA hydroxysteroid dehydrogenases produced by intestinal *Bacteroides*, Firmicutes (including *Clostridium*, *Eubacterium*, and *Ruminococcus*), and *Escherichia* (60).

BAs are endocrine signaling molecules that affect host physiology *via* the activation of BA receptors. The two major BA receptors that regulate the host metabolism are the nuclear farnesoid X receptor (FXR) and the membrane-bound Takeda G protein-coupled receptor 5 (TGR5) (62). Both FXR and TGR5 have protective roles in DKD (63).

Renal expression of FXR is predominantly tubular and less prominently glomerular, mesangial, and podocytal (64). FXR expression is decreased in people with diabetes- and obesity-related kidney disease. In a series of rodent models of diabetes, the expression levels of FXR and its target genes were found to be downregulated in the kidney (65). Supplementation with FXR agonists, such as tauroursodeoxycholic acid, has been shown to attenuate glomerular and tubular injury in db/db mice and diabetic endothelial nitric oxide synthase-deficient mice (66). Moreover, the FXR agonist GW4064 can improve the functional and structural changes in the kidney of db/db mice (67).

TGR5 expression and activity is impaired in the kidneys of humans and rodents with obesity and diabetes (68). TGR5

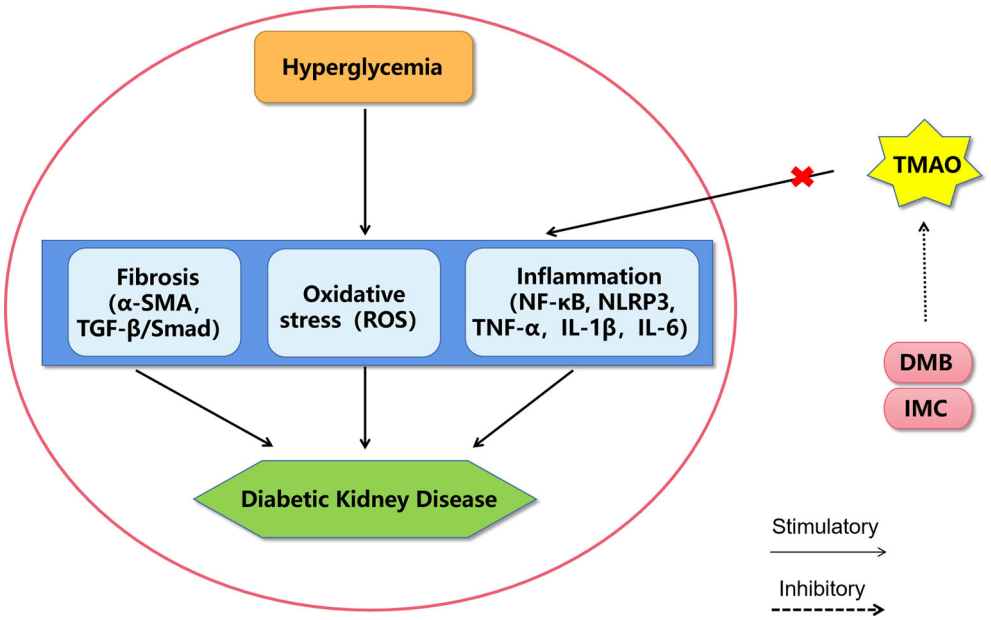

FIGURE 1 | A proposed model of diabetic kidney disease mediated by TMAO. The mechanism of TMAO promoting the progression of diabetic kidney disease may be through promoting inflammation, oxidative stress and fibrosis in renal system. The choline TMA lyase inhibitor DMB and IMC may improve diabetic kidney disease by inhibiting TMAO levels. DMB, 3,3-dimethyl-1-butanol; IMC, iodomethylcholine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, Nod-like receptor pyrin domain 3 inflammasome, TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IL-6, interleukin-6; ROS, reactive oxygen species; α-SMA, alpha sarcomeric actin; TGF-β, transforming growth factor-β.

activation reduces the renal inflammatory reactions in diabetic mice, thereby improving renal fibrosis (69). In high glucose-treated glomerular mesangial cells, TGR5 activation was found to significantly decrease the expression levels of transforming growth factor beta 1 and fibronectin, which can both accelerate renal fibrosis (70, 71).

The BA signaling pathway plays an extremely important role in T2D and DKD, and it is an important target for drug intervention (**Figure 2**). BAs have been used directly to treat diabetes and obesity. Metformin, which is a first-line antidiabetic drug, acts in part through the intestinal FXR axis to improve T2D. Oral synthetic FXR antagonists may be of potential translational value in the clinical treatment of T2D (72) Moreover, both BA sequestrants and apical sodium-dependent BA transporter inhibitors can reduce BA absorption and have a therapeutic effect on T2D by activating FXR (73). In the future, semisynthetic BA analogues for the treatment of T2D and DKD need more focus.

Protein-Bound Uremic Toxins

Protein-bound uremic toxins, such as indoxyl sulfate (IS), p-cresyl sulfate (pCS), p-cresyl glucuronide (pCG), and phenyl sulfate, originate from the gut microbial metabolism of the aromatic amino acids, tyrosine, phenylalanine, and tryptophan. These uremic toxins have been associated with cardiovascular disease and mortality in CKD, and several uremic toxins have also been found to exert toxic effects in the kidney. The levels of these uremic toxins are elevated in T2D patients who progress to end-stage kidney disease (74, 75) and elevated levels of these uremic toxins increase the risk of progression to end-stage kidney disease in patients with T2D (74).

IS is derived from tryptophan metabolism. Tryptophan is digested by intestinal bacteria (*E. coli*, *Proteus vulgaris*, *Paracolobactrum coliforme*, *Achromobacter liquefaciens*, and *Bacteroides* spp) to indole, and it is metabolized to IS in the liver (76). Increasing levels of IS are correlated with changes in albuminuria and the estimated glomerular filtration rate, and they are associated with the progression of DKD in patients with T1D and T2D (77–79), as well as in animal models of DM (80–82). IS also can directly induce tubulointerstitial injury, renal oxidative stress and inflammation in mice that undergone nephrectomy (83, 84), as well as in human renal proximal tubular epithelial (HK-2) cells (85, 86).

Both pCS and pCG originate from the intestinal microbial fermentation of tyrosine into p-cresol, and p-cresol is subsequently conjugated to either sulfate or glucuronic acid resulting in the formation of pCS or pCG, respectively (87). The intestinal bacteria generating p-cresol mainly belong to the families Bacteroidaceae, Bifidobacteriaceae, Clostridiaceae, Enterococcaceae, Eubacteriaceae, Fusobacteriaceae, Lachnospiraceae, Lactobacillaceae, Porphyromonadaceae, Staphylococcaceae, Ruminococcaceae, and Veillonellaceae (88). The levels of pCS and pCG are elevated in patients with CKD, and pCG can cause phenotypical changes in renal proximal tubule cells (89). In addition, pCS can directly influence cell viability and induce cell death (90, 91). It has also been reported that pCS can induce reactive

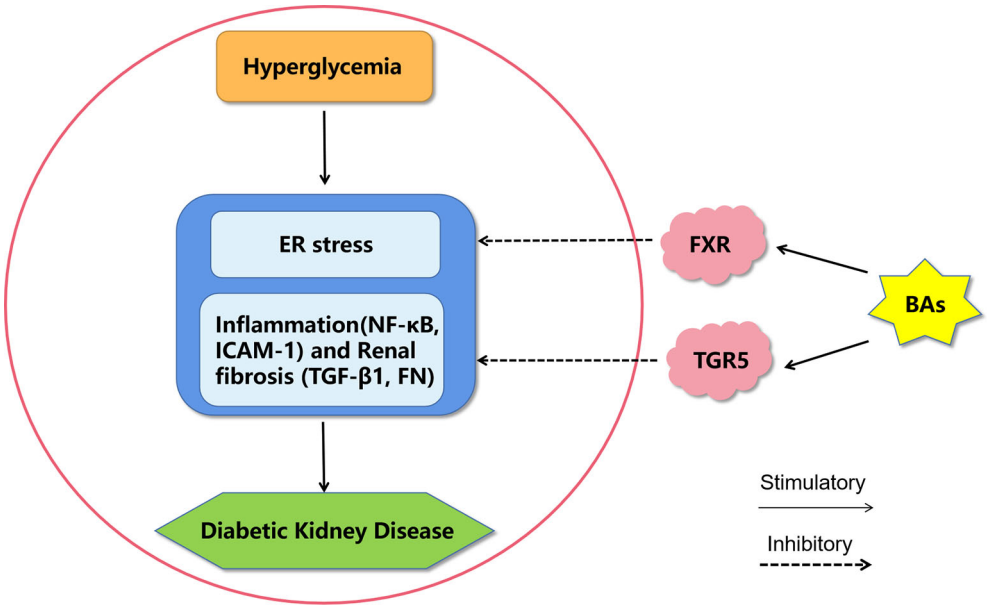

FIGURE 2 | A proposed model of diabetic kidney disease mediated by bile acids. Bile acids may inhibit endoplasmic reticulum stress, inflammation and fibrosis by activating FXR and TGR5 to improve diabetic kidney disease. BAs, bile acids; FXR, Farnesoid X receptor; TGR5, G protein-coupled receptor 5; ER stress, endoplasmic reticulum stress; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ICAM-1, intercellular adhesion molecule-1; TGF-β1, transforming growth factor-β1; FN, fibronectin.

oxygen species and inflammatory cytokines in 5/6 nephrectomized rats and HK-2 cells (92).

Phenyl sulfate is produced by the metabolism of the amino acid tyrosine by the gut microbiota. First, phenol is synthesized in the gut and then metabolized into phenyl sulfate by the liver. The intestinal bacteria generating phenol mainly belong to the families Clostridiaceae, Enterococcaceae, Staphylococcaceae, Bacteroidaceae, Bifidobacteriaceae, and Enterobacteriaceae (93, 94). Phenyl sulfate is then secreted by proximal tubular cells through the action of SLCO4C1, which is the only organic acid transporter polypeptide in the human kidney (95). In a cohort of diabetic patients, phenyl sulfate levels were found to be statistically significantly correlated with both basal albuminuria and the 2-year progression of DKD. Further, phenyl sulfate was found to directly induce albuminuria *via* podocyte damage in diabetic animal models (15).

These protein-bound uremic toxins are not only markers of the risk for DKD occurrence in diabetic patients, but they are also risk factors that directly facilitate the development of DKD (**Figure 3**). However, the molecular mechanisms of these uremic toxins in DKD still need further study. Some drugs that target uremic toxins, such as AST-120 (Kremezin), which is an oral adsorbent, protect against the progression of both DKD (84, 96) and CKD (97, 98) by removing serum and urinary uremic toxins.

Polyphenols-Derived Microbial Metabolites

Polyphenols are produced in plants and have excellent antibacterial, antifungal, antioxidant, and photo-protective properties (99). Natural polyphenols, such as ellagitannins, lignans, isoflavones, and flavanones, which are poorly absorbed or not absorbed at all. Interaction with gut microbiota leads to the biochemical transformations of the native phytochemicals into more bioavailable metabolites. These gut microbiota transformations are grouped into three major catabolic processes: hydrolysis (O-deglycosylations and ester hydrolysis), cleavage (C-ring cleavage; delactonization, demethylation), and reductions (dehydroxylation and double bond reduction) (100). *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterococcus*, *Enterobacter*, and Firmicutes have been demonstrated to participate in hydrolysis (101–103). Cleavage reactions are catalyzed by *Clostridium*, *Coriobacteriaceae*, *Eubacterium* and *Eggerthella* strains (101, 104). *Gordonibacter urolithinfaciens* and *Lactonifactor longoviformis* catalyze different reduction reactions (105–107).

Enterolactone and enterodiol, which are therapeutically relevant polyphenols, are formed as the secondary gut bacterial metabolites of lignans. Urinary levels of enterolactone are associated with lower risk of developing T2D in women in the United States (108). Pre-diagnostic enterolactone concentrations are inversely associated with all-cause and diabetes-specific mortality (109). Enterolactone was found to increase glucose uptake in an AMPK-dependent manner in L6 myotubes and to improve glucose tolerance in db/db mice (110). Many studies have reported that enterolactone may have some benefit for T2D. However, there are also some opposite conclusions. One study did not find a significant association between urine enterolactone levels and T2D risk in Chinese adults (111). Moreover, enterolactone was found to enhanced the hepatic insulin resistance *via* increased sphingolipid concentrations in the

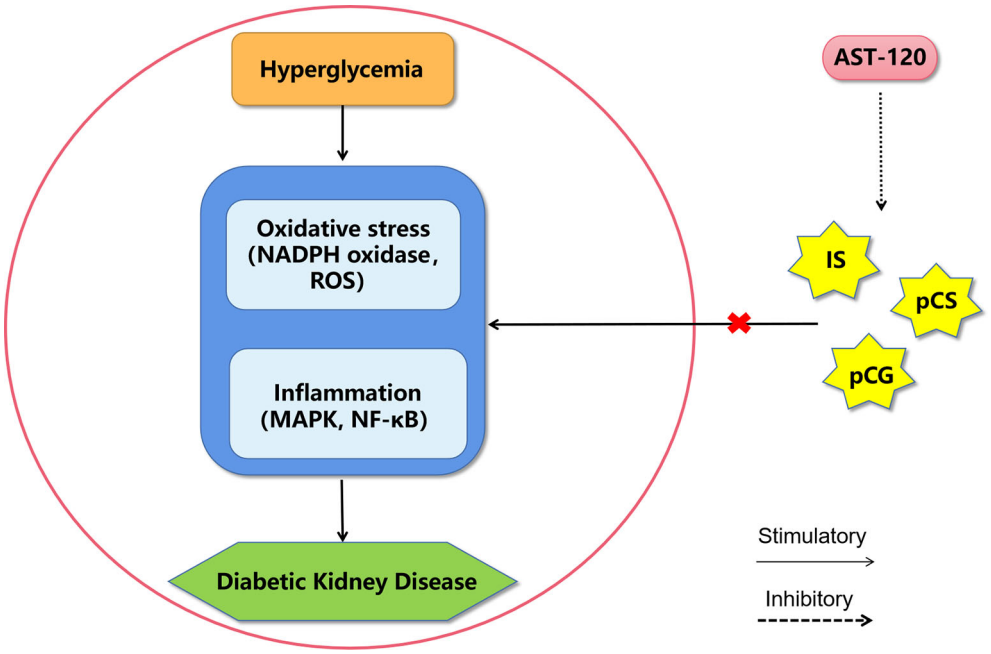

FIGURE 3 | A proposed model of diabetic kidney disease mediated by protein-bound uremic toxins. The mechanism of protein-bound uremic toxins promoting the progression of diabetic kidney disease may be through promoting inflammation and oxidative stress in renal system. AST-120 may improve diabetic kidney disease by removing serum and urinary uremic toxins. IS, Indoxyl sulfate; pCG, p-cresyl glucuronide; pCS, p-cresyl sulfate; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

palmitate-rich condition of HepG2 cells (112). Thus, the relationship between enterolactone and T2D should be further studied.

Urolithin A (UA), which is a main gut microbiota-derived metabolite of pomegranate ellagitannins, plays a direct role in improving systemic insulin sensitivity (113). In addition, UA can prevent high-fat-diet-induced insulin resistance and glucose intolerance in mice (114). Further, UA can effectively improve β-cell dysfunction, possibly by regulating autophagy and the AKT/mTOR signaling pathway in the pancreas of diabetic mice (115). Moreover, UA has shown a protective effect in some acute kidney injury animal models *via* modulation of inflammation, oxidative stress, autophagy, and apoptosis (52, 116–118), which are also some of the main mechanisms of DKD. Thus, UA may be capable of attenuating DKD. Urolithin C (UC), which is another microbiota ellagitannin metabolite, is a glucose-dependent activator of insulin secretion *via* the facilitation of the opening of L-type Ca^{2+} channels in β-cells (119). UC can also influence β-cell function by affecting the activation of intracellular signaling proteins, specifically ERK1/2 (120). Urolithin also has been reported have some certain benefits for diabetes complications. Urolithins (includes urolithin A, B, C, D and urolithin B-3-O-glucuronide) may exert positive effects in modulating the pro-inflammatory mediators and growth factors by rat cardiac myocytes and fibroblasts exposed to high glucose concentrations (121). In streptozotocin-induced diabetic rat, both urolithin A and B administration may be able to prevent the initial inflammatory response of myocardial tissue to hyperglycemia and the negative impact of the altered diabetic milieu on cardiac performance (122).

Taken together, polyphenols might have differential microbial metabolites that may improve T2D and its complications. Thus, it is crucial to understand the bacterial pathways involved in the metabolism of polyphenols and their specific roles in T2D and DKD.

Branched-Chain Amino Acids

BCAAs, including leucine, isoleucine, and valine, are among the nine essential amino acids synthesized by the gut microbiota. The food sources most enriched in BCAAs are meat, fish, dairy products, and eggs (123).

BCAAs are known as biomarkers for insulin resistance and predictors of diabetes development (124). In a recent prospective cohort study, BCAAs showed a strong association with early risk of T2D (125). The serum metabolomes of insulin-resistant and T2D individuals are characterized by increased BCAAs levels (126). Higher intake of total dietary BCAAs, leucine and valine in particular, may increase the incidence of insulin resistance by more than 60% in adults and play an important role in the development of diabetes (127). In contrast, short-term reduction of dietary BCAAs may acutely decrease meal-induced insulin secretion, and improve postprandial insulin sensitivity (128). Furthermore, in both animal and cell models, excess BCAAs have been found to result in liver insulin resistance (129, 130). These results suggest that BCAAs may not only be biomarkers, but also causal agents of insulin resistance and T2D. Evidence of

a causal role of BCAAs in human diabetes has also been suggested in some genetic studies. In a mendelian randomization analysis, researchers used genome-wide association studies coupled with large-scale metabolomic measurements to investigate the aetiologic relationship between BCAA metabolism and T2D. They suggested that BCAA-raising polymorphisms were associated with a higher risk of T2D (131). In addition, genetic evidence suggests that genetically elevated insulin resistance is associated with higher concentrations of all BCAAs, supporting the idea that BCAA metabolism lies on a causal pathway from adiposity and insulin resistance to T2D (132). Another study showed that higher BCAA levels do not have a causal effect on insulin resistance while increased insulin resistance drives higher fasting levels of circulating BCAAs (133).

In T2D patients with stages 1 or 2 CKD, high serum BCAA levels are independently associated with a decline in the estimated glomerular filtration rate (134). BCAAs can also directly influence renal function. In one animal model, 5/6 nephrectomized rats receiving a BCAA diet showed a decrease in the estimated glomerular filtration rate and an increase in smooth muscle actin and collagen mRNA expression levels, suggesting renal dysfunction, greater inflammation, and fibrosis in the kidney (135).

However, several studies have suggested that BCAAs may be an effective means of preventing and treating DM and DKD. Moderate intake of BCAA-rich protein was found to improve glucose homeostasis in mice fed a high-fat diet (136). In streptozotocin-induced diabetic rats, treatment with a low dose of BCAAs was found to recover islet function (137). Moreover, one study found that BCAAs countered oxidative stress in the kidney of diabetic rats and alleviated diabetic kidney injury (138), while another study showed that BCAAs protected renal mesangial cells from high-glucose-induced stress (139). More studies are needed to clarify the relationships between BCAAs and T2D. Furthermore, the factors resulting in the differences among these studies needed to be identified.

Many bacterial species are capable of regulating biosynthesis, transport, and metabolism of BCAAs (140). Moreover, transplantation of the microbiota from obese humans to germ-free mice causes a significant increase in circulating BCAAs (141). Additional studies are needed to quantify the microbiota-derived BCAAs in the circulating pool of these metabolites.

Other Metabolites

Imidazole propionate, which is a microbially produced amino acid-derived metabolite, is present at higher concentrations in people with T2D. A study demonstrated that imidazole propionate directly impaired glucose tolerance and insulin signaling through mTORC1, meaning that imidazole propionate may contribute to the pathogenesis of T2D (142).

4-Cresol is a product of the colonic fermentation of tyrosine and phenylalanine, and it has been reported to be related to T2D. Serum concentrations of 4-cresol are inversely correlated with T2D. The chronic administration of nontoxic doses of 4-cresol was found to reduce adiposity and glucose intolerance in an animal model, and 4-cresol was found to stimulate insulin secretion and β-cell proliferation *in vivo* and *in vitro* (143, 144). 4-Cresol may be a regulator of T2D endophenotypes and have potential therapeutic applications.

The metabolism of dietary and host-derived sulfur-containing compounds to hydrogen sulfide (H_2S) by the gut microbiota has many prominent connections to host health and disease (145). H_2S potentially has both beneficial and toxic effects (146). The plasma H_2S levels of Japanese patients with T2D were found to be reduced and significantly associated with their hemoglobin A1c levels (147). H_2S also has been suggested to be linked with GLP-1, which has become an important therapeutic target in the treatment of T2D and obesity. By supplementing with a prebiotic chondroitin sulfate, mice were found to enhanced GLP-1 and insulin secretion, improved oral glucose tolerance, and reduced food consumption, suggesting that H_2S plays a stimulatory role in GLP-1 secretion (148). However, chronic administration of H_2S-releasing agents was found to increase serum glucose, decrease glucose tolerance, and decrease insulin secretion in rats with T2D (149). In the future, studies are needed to clarify the effects of different doses of H_2S in T2D. As for DKD, H_2S has been reported to have a protective effect and may be used as a novel therapeutic agent.

Hippuric acid is a gut microbial mammalian co-metabolite of benzoic acid; it is subsequently conjugated with glycine in the mitochondria, and then excreted in the urine (150). Several metabolomics studies have shown that hippuric acid levels in patients with impaired glucose tolerance and diabetes are lower than those in healthy people (151). Hippuric acid has also been proposed as a potential urinary biomarker for fruit, vegetable, and polyphenol consumption. Consumption of bilberries and flavonoids, which can increase hippuric acid levels, is associated with a favorable risk factor profile for T2D and better glucose and insulin metabolism (152, 153). Further, hippuric acid has been reported to be decreased in both human diabetic renal pathology studies (154) and animal DKD models (155), and it has been suggested as an additional indicators of DKD. However, there is no evidence to excluded the direct effect of hippuric acid on T2D or DKD. Further studies are needed to infer causality between hippuric acid and T2D or DKD.

THERAPEUTIC DRUGS FOR DKD AND GUT MICROBIAL METABOLITES

In general, none of the widely used current treatments specifically address the underlying molecular processes responsible for DKD. Several interventional strategies have involved multifactorial approaches, including blood pressure and glucose lowering (156).

RAAS inhibitors have been the treatment of choice for DKD, following the publication of clinical trial results demonstrating benefits of angiotensin converting enzyme inhibitors and angiotensin receptor blockers for decreasing albuminuria in patients with DKD (157). Metformin, the most frequently administered medication to decrease blood glucose, has recently been suggested to enrich SCFA-producing microbiota, such as

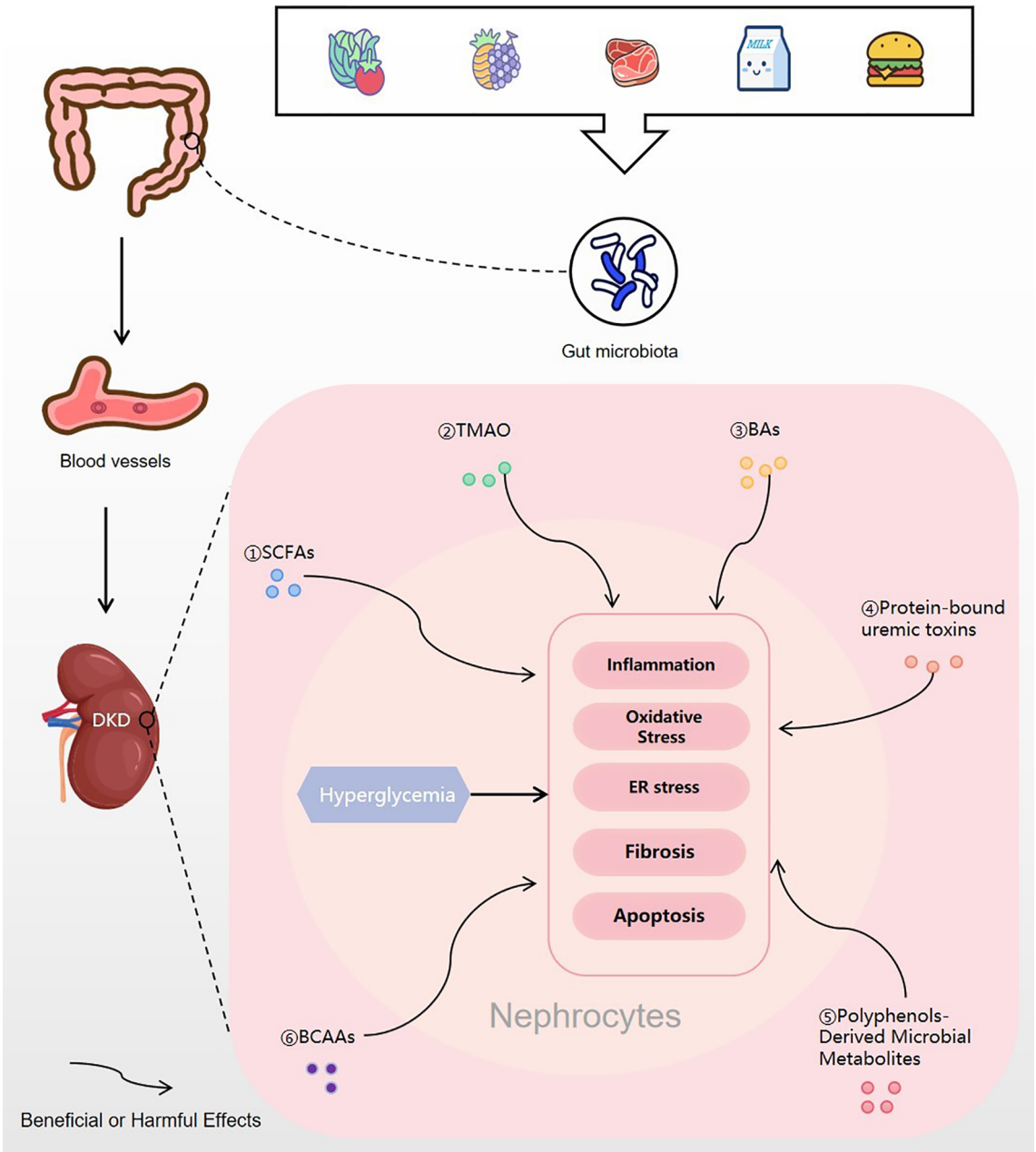

FIGURE 4 | Gut microbial metabolites regulating DKD. Food is digested and absorbed by the gastrointestinal tract, and various gut microbial metabolites are produced under the action of the gut microbiota. Then, the microbial metabolites are absorbed into the blood vessels and finally enter the kidney. In nephrocytes, gut microbial metabolites of SCFAs, TMAO, BAs, protein-bound uremic toxins, polyphenols-derived microbial metabolites and BCAAs may trigger or inhibit inflammation, oxidative stress, ER stress, fibrosis or apoptosis, which will improve or excacerbate the progress of DKD. DKD, diabetic kidney disease; SCFA, short-chain fatty acid; TMAO, trimethylamine N-oxide; BA, bile acids; BCAA, branched-chain amino acid; ER stress, endoplasmic reticulum stress.

such as *Blautia*, *Bacteroides*, *Butyricoccus*, *Bifidobacterium*, *Prevotella*, *Megasphaera*, *Butyrivibrio* (158). SGLT2 inhibitors, GLP-1 receptor agonists, and dipeptidyl peptidase 4 (DPP4) inhibitors are three new classes of glucoselowering agents for patients with DKD (159–161). SGLT2 inhibition has been reported to promote elevations in the levels of the SCFAs acetate and butyric acid in cecal contents of hypertensive mice (162). Vildagliptin, a DPP4 inhibitor, has been reported to enrich SCFA-producing bacteria (163). Moreover, mice on HFD with DPP-4 inhibitor PKF-275-055 treatment showed enriched butyrate-producing *Rumminococcus* and of the acetogen *Dorea* (164).

Some drugs for the treatment of DKD can increase the level of SCFA by enriching SCFA-producing microbiota. Whether the

mechanism of these drugs in the treatment of DKD is mediated by gut microbial metabolites remains unknown. The relationship between these drugs and gut microbial metabolites still needs more research.

CONCLUSIONS AND FUTURE PERSPECTIVES

There is growing evidence of the roles of gut microbial metabolites as biomarkers of the pathophysiological features or as pathogenic agents of DKD. In this review, we highlighted aspects relating to the involvement of microbiota metabolites in the pathogenesis of DKD (**Figure 4**). In the future, we should continue to look for microbial metabolites that can be used to diagnose and treat DKD. However, there are still many challenges to be overcome on the path toward using microbial metabolites for therapies.

First, most microbial metabolites act as signaling molecules by binding to receptors and triggering downstream signaling cascades. Therefore, we should identify the targets of these microbial metabolites in humans. Some metabolites, such as SCFAs and BAs, interact with G-protein-coupled receptors (GPCRs) associated with diverse functions. Many studies have shown that large-scale functional screening for GPCRs can identify microbial metabolites that exert various physiological functions by activating GPCRs. There are many undiscovered receptors similar to GPCRs, and efforts should be made to identify these receptors and generate drugs targeting these receptors in host tissues.

Second, most studies to date have only clarified the correlations between microbial metabolites and T2D, and lack of research on causality. Large prospective cohort studies are needed to determine if microbial metabolites are altered prior to or after disease onset. The result of some studies showing causal effects in rodents should be confirmed in humans. If the results on causality can be confirmed in humans, then further research on the human intestinal microbiota may lead to the development of novel diagnostic and therapeutic tools.

Finally, the concentrations of intestinal metabolites might be highly context-dependent. If the metabolite is beneficial to human health, it needs to be supplemented once its level falls below the physiological level. The supplementation strategy needs to consider the route and frequency of administration, individual differences in pharmacokinetics, and side effects for doses that exceed physiological concentrations. If the metabolite contributes to the pathophysiology of the disease, then the production of the metabolite needs to be suppressed. Inhibiting related enzymes that produce metabolites is a promising method. Developing inhibitors targeting bacterial enzymes is another therapeutic strategy to prevent the action of harmful microbial metabolites, as in the case of TMA lyase producing TMA, a precursor for TMAO. Metabolites do not act in isolation, and thus the combined signals mediated by different metabolites need to be investigated.

We believe that numerous challenges must be overcome on the path toward using microbial metabolites for therapies. Despite these difficulties, the metabolic pathways involved in the production and signaling of microbiome-related metabolites are huge untapped opportunities to regulate disease susceptibility.

AUTHOR CONTRIBUTIONS

QF, NL, and BZ performed the literature search. QF drafted the manuscript. XZ, FG, and XH provided critical intellectual contributions. DO directed the research and made the critical revision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Gut Microbiota: The Missing Link Between *Helicobacter pylori* Infection and Metabolic Disorders?

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Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that infects approximately 4.4 billion individuals worldwide. Although the majority of infected individuals remain asymptomatic, this bacterium colonizes the gastric mucosa causing the development of various clinical conditions as peptic ulcers, chronic gastritis and gastric adenocarcinomas and mucosa-associated lymphoid tissue lymphomas, but complications are not limited to gastric ones. Extradigestive pathologies, including metabolic disturbances such as diabetes, obesity and nonalcoholic fatty liver disease, have also been associated with *H. pylori* infection. However, the underlying mechanisms connecting *H. pylori* with extragastric metabolic diseases needs to be clarified. Notably, the latest studies on the topic have confirmed that *H. pylori* infection modulates gut microbiota in humans. Damage in the gut bacterial community (dysbiosis) has been widely related to metabolic dysregulation by affecting adiposity, host energy balance, carbohydrate metabolism, and hormonal modulation, among others. Taking into account that Type 2 diabetic patients are more prone to be *H. pylori* positive, gut microbiota emerges as putative key factor responsible for this interaction. In this regard, the therapy of choice for *H. pylori* eradication, based on proton pump inhibitor combined with two or more antibiotics, also alters gut microbiota composition, but consequences on metabolic health of the patients has been scarcely explored. Recent studies from our group showed that, despite decreasing gut bacterial diversity, conventional *H. pylori* eradication therapy is related to positive changes in glucose and lipid profiles. The mechanistic insights explaining these effects should also be addressed in future research. This review will deal with the role of gut microbiota as the linking factor between *H. pylori* infection and metabolic diseases, and discussed the impact that gut bacterial modulation by *H. pylori* eradication treatment can also have in host's metabolism. For this purpose, new evidence from the latest human studies published in more recent years will be analyzed.

Keywords: *Helicobacter pylori*, gut microbiota, metabolism, eradication therapy for *Helicobacter pylori*, metabolic diseases, diabetes

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic spiral-shaped Gram-negative bacterium that can colonizes the stomach. This bacterium is able to generate an alkaline microenvironment that allows its survival in the acid gastric environment (1). The prevalence of *H. pylori* infection is more than 50% worldwide, but ranges from 19 to 88% depending on geographical area localization, with a higher prevalence in developing than in developed countries (2), which has been related to socioeconomic status and hygiene levels, including level of urbanization, sanitation, and access to clean water (2). However, other factors such as racial group could also be related to *H. pylori* prevalence (2, 3). The high prevalence of this infectious disease is a matter of concern due to the high number of *H. pylori*-associated clinical conditions.

H. pylori infection disturbs gastric homeostasis and, although most of the infected individuals remain asymptomatic, serious gastrointestinal complications are attributed to this bacterium such as peptic ulcer or chronic gastritis and promotes other life-threatening complications such as gastric cancer and mucosa-associated lymphoid tissue lymphomas (4). *H. pylori*-related diseases are not only restricted to the gastrointestinal tract, but *H. pylori* infection has also been associated with a number of extra-gastroduodenal disorders. In fact, there are solid evidence on its relationship with hematological disorders such as idiopathic thrombocytopenic purpura, iron deficiency anemia and vitamin B12 deficiency (5), but there is also a growing body of evidence that supports a link with neurological (e.g. Parkinson or Alzheimer disease, age-related cognitive decline), cardiovascular, liver, metabolic (e.g. diabetes, obesity, metabolic syndrome) and autoimmune and inflammatory (e.g. autoimmune gastritis, immune thrombocytopenia purpura, autoimmune thyroid diseases, inflammatory bowel diseases) disorders (6).

The precise mechanisms underlying the connection between gastric *H. pylori* infection and extra-gastroduodenal diseases remain unclear. The most accepted hypothesis to date is the generation of an inflammatory milieu due to the *H. pylori* insults to gastric mucosa and the consequent activation of innate and adaptive responses (1, 4). It has been hypothesized that this local inflammation at the stomach can spread systematically by the release of proinflammatory cytokines. This would favor the establishment of a low-grade and chronic inflammation, that is a common feature of *H. pylori*-associated extra-gastroduodenal disorders including cardiometabolic diseases such as diabetes, atherosclerosis or dyslipidemia (7, 8). However, this hypothesis has not been formally confirmed as yet, and knowledge about how inflammation actually links *H. pylori* with metabolic disorders is still insufficient.

Within this context, recent evidence showing that *H. pylori* infection not only disturbs the equilibrium of commensal bacterium species in the gastric mucosa, but it also leads to microbial changes in the human gut (9–18), has brought gut microbiota into the limelight (7, 9). Gut microbiota participates in host's immune and metabolic homeostasis. In fact, dysbiosis of the gut bacterial community has been widely associated with obesity, diabetes and metabolic syndrome (19–21). Then, modulation of the gut microbiota during *H. pylori* infection could be triggering the onset and the impairment of metabolic disorders. Nevertheless, human studies addressing this issue are still scarce (10, 22–24).

Prophylactic recommendations to prevent *H. pylori* infection such as good household hygienic practices and the use of safe supplies of clean water are not always feasible, and while awaiting for an efficient *H. pylori* vaccine, the antibiotic therapy is the strategy of choice to fight against *H. pylori* infection at present (25, 26). Therapies for *H. pylori* eradication are based on the use of different antibiotic combinations (27–29) which are well known to have profound effects on the composition and diversity of the gut microbiota (30) and might also be related to adiposity and insulin resistance (31–34). Although an increasing number of studies have emerged analyzing the effects of the various antibiotic eradication therapies for *H. pylori* eradication on the composition of the gut microbiota (10, 11, 16, 17, 24, 35–44), few of them have related these changes with patients' metabolic traits (10, 22–24). Within this context, our group has recently focused on analyzing how gut microbial changes promoted by *H. pylori* and its treatment can relate to metabolic traits.

In this review, we discuss the latest evidence from human studies on the influence of *H. pylori* infection and different eradication therapies on the composition of the gut microbiota, with particular focus on the relationship between *H. pylori*-induced gut microbiota modifications and host's metabolic health.

H. PYLORI INFECTION AND METABOLIC DISEASES

Among the *H. pylori* infection-derived extra-gastric manifestations, the link with energy management, weight gain and metabolic homeostasis is still up for debate (6).

On the one hand, alterations in the gastrointestinal microenvironment due to *H. pylori* infection have been suggested to impair nutrient absorption and causes micronutrient deficiency. Malabsorption of iron and vitamins such as vitamin B12, vitamin A, vitamin C, vitamin E or folic acid have been related to *H. pylori* infection. Concordantly, associations between micronutrient deficiency complications and *H. pylori* infection have been found. For instance, the incidence of iron and B12 deficiency anemias is higher in *H. pylori* positive patients than in non-infected subjects (45, 46). Other disorders related to micronutrient deficiencies have been suggested to be associated with *H. pylori* infection, although further evidence would be necessary to confirm this hypothesis (45, 47, 48).

Moreover, lower levels of the orexigenic gastric hormone ghrelin (49), and higher levels of the anorexigenic adipokine leptin (50) have been reported in *H. pylori* infected patients. All together, these data led to propose that *H. pylori* infection can cause growth retardation and malnutrition in children. However, studies found that growth delay was also dependent on socioeconomic factors (8). Findings on weight management in adults during *H. pylori* infection are controversial with studies having reported positive (51–53), negative (54, 55) or non

correlation (56, 57) between *H. pylori* infection and increased body mass index (BMI).

H. pylori eradication has been associated with weight gain likely due to the improvement of postprandial dyspeptic symptoms. In addition, changes in ghrelin and leptin levels after *H. pylori* treatment could be also acting on weight increase (58–60). An intervention study carried out in *H. pylori* positive children with previous growth retardation and low ghrelin levels, showed restoration of normal ghrelin levels and increased weight gain upon *H. pylori* eradication (61). By contrast, other studies reported significant weight loss, decrease in fat mass percentage and increase in fat-free mass after *H. pylori* eradication treatment (62, 63) or no effect on body weight (64) in adults. These contradictory results can be due to the different populations analyzed including different age groups. The influence of *H. pylori* treatment on body weight seems to differ in adults and children which could be due to age-related phenomena on body growth. In addition, other factors, such as gut microbiota, could be determining these discrepant results as discussed below.

On the other hand, imbalance of the aforementioned and other hormones such as glucagon-like peptide 1 (GLP-1), and the generation of a proinflammatory milieu might negatively affect metabolic homeostasis and weight in the adulthood (8). In fact, low-grade inflammation is a common feature of obesity, diabetes, insulin resistance, dyslipidemia and cardiovascular diseases (65).

Patients suffering of *H. pylori* infection are prone to display an unfavorable pro-atherogenic lipid profile featured by high triglycerides, total cholesterol and LDL-C and decreased HDL-C levels (22, 66–69). Zhao et al., found that, in addition to the dyslipidemic profile, bilirubin levels were also diminished in *H. pylori* infected patients (70). Bilirubin exhibits powerful antioxidant properties and has been inversely related to the risk of cardiovascular diseases and metabolic syndrome (71, 72). However, some studies did not find correlations between *H. pylori* infection and some of these lipid variables (73, 74). A recent meta-analysis of 27 previous studies concluded that most of the evidence tends towards an unfavorable lipid profile in *H. pylori* infected patients, although this is still casting doubt on the precise relationship with triglyceride levels (75). The causes of the altered lipid profile have not been elucidated, but impaired intestinal absorption and altered bile acid dynamics might be contributing to this condition (76). Notably, as discussed below, gut microbiota modulates these processes and could be acting as mediators in the establishment of the dyslipidemic profile in *H. pylori* patients. Supporting this hypothesis, antibiotic treatment for *H. pylori* eradication resulted in total and LDL cholesterol reduction and increase in HDL-C levels (77). Other studies described an elevation in total cholesterol levels at the expense of increasing HDL-C after *H. pylori* eradication (22, 78). However, the lipid improvement after *H. pylori* eradication was not confirmed by other authors (74, 78, 79). If considered the gut microbiome hypothesis, factors that can differentially modulate the gut microbial composition such as diet or the precise antibiotic combination used, might account for these discrepant results.

Associations between *H. pylori* infection and glucose homeostasis have been also explored (10, 23, 80, 81). Evidence suggests that *H. pylori* may be involved in both diabetes onset and impaired glycemic control in diabetic patients, but contradictory trends or lack of association between *H. pylori* infection and type 2 Diabetes have been also reported (80, 81). One of the mechanisms proposed for this relationship is the proinflammatory milieu induced by *H. pylori* infection that promotes gastric inflammation and cytokine secretion. In fact, *H. pylori* infection has been associated with increased levels of C-reactive protein (CRP) and interleukin 6 (IL6) (82, 83).

The link between *H. pylori* and Type 2 Diabetes was first analyzed in 1989 by Simon et al., who found that the prevalence of *H. pylori* infection was higher in patients with Type 2 Diabetes compared to asymptomatic volunteers (84). Thenceforth, most of the studies have corroborated this finding reporting a higher prevalence of *H. pylori* in patients with Type 2 Diabetes (usually greater than 50%) in diabetic patients than in non-diabetic subjects (85–88). Recently published meta-analyses this year aimed at elucidating the direction of the relationship between *H. pylori* and Type 2 Diabetes (81). Pooled *H. pylori* prevalence in diabetic patients was 54%, but there were high (even contradictory) regional variability as the highest prevalence of *H. pylori* infection in patients with T2DM was 66% in Africa and the lowest was 15% in USA (81). Moreover, it was found that patients with *H. pylori* infection had a higher risk of Type 2 Diabetes, but results again differed depending on geographical regions with a direct relationship in Europe, Asian and Africa, but a negative relationship in USA (80). In subgroup analysis, the relationship between *H. pylori* and the risk of diabetes was different according to age, level of glycated hemoglobin A (HbA1c), duration of diabetes and methods for *H. pylori* detection. This suggests that these factors could be an important source of heterogeneity in the studies included in the meta-analyses (80, 81).

Furthermore, there are evidence suggesting that *H. pylori* infection is related to worse outcomes in diabetic patients. For instance, HbA1c levels were higher in diabetic patients with *H. pylori* infection in comparison with diabetic subjects negative for *H. pylori* (89). On the other hand, Yang et al. concluded that diabetic patients with *H. pylori* infection showed higher risk of cardiovascular diseases and more severe peripheral arterial stiffness than diabetic patients without *H. pylori* infection (90). Reciprocally, diabetic *H. pylori* positive patients showed worse symptomatology related to the infection (91, 92). A higher degree of insulin resistance was also reported in *H. pylori* infected patients from different populations (8), an association that was confirmed in every studies included in a systematic review but one (88).

Therefore, although the link between *H. pylori* infection and Type 2 Diabetes has been controversial in some studies, the general trend suggests a higher susceptibility to *H. pylori* infection in diabetic patients. Likewise, *H. pylori* infection would promote the development of Type 2 Diabetes, as well as worse glycemic control and insulin responsiveness, likely due in part to the enhanced inflammation. In this line, gut microbiota could be a contributing factor as a modulator of the inflammatory response

as well as of the secretion of incretins involved in glucose homeostasis as detailed in next section. In this regards, some clues have been given from intervention studies based on the use of a cocktail of antibiotics for *H. pylori* eradication and found that non-diabetic patients with *H. pylori* infection subjected to antibiotic treatment, experienced an improvement in insulin resistance index, HbA1c and insulin levels concomitant to a reduction of low-grade inflammation (10, 87, 93). Of note, efficiency on eradication rates of infection was lower in diabetic patients than in non-diabetic (8). Higher rates of antibiotic resistance in diabetic patients than in non-diabetic ones might account for these differences (94). Furthermore, the improvement in glucose homeostasis, i.e. HbA1c and glucose levels, in diabetic patients after successful eradication of *H. pylori*, were not statistically significant in comparison with non-diabetic controls or baseline values (95). Notably, gut microbiota composition varies in diabetic patients as compared to non-diabetic subjects (96–98).

GUT MICROBIOTA, ANTIBIOTIC THERAPY, AND METABOLIC DISEASES

The human gut is colonized by a myriad of microorganisms that encompass bacteria, fungi, archaea, protozoa and viruses. This complex ecological community comprises symbiotic, commensal and pathogenic microbes that physiologically interact with the host at different levels and functions. Locally, gut microbiota contributes to the maintenance of gut barrier function and integrity, fermentation of indigestible dietary substrates, vitamin synthesis and immune system regulation. However, the activity of these microorganisms goes beyond the gastrointestinal tract, affecting the function of distant organs including brain, liver, pancreas, muscle or adipose tissue (99).

The impact that gut microbiota has on host's metabolism is perhaps one of the most evident interrelations between the two systems. Besides harvesting energy from indigestible dietary fibers and modulating enterocyte function, gut bacterial community can also indirectly affect metabolic homeostasis by regulating immune system, local hormone secretion or bile acid synthesis. The efficiency of these actions depends on the specific and prevailing bacterial species that reside in the intestine (20, 100).

Several reports described differential bacterial profile in the gut from both obese and diabetic subjects (96–98). Although there are some controversial studies, in general terms, obesity-associated microbiota is depicted by an increase in *Actinobacteria* and *Firmicutes* and decline in *Bacteroidetes* phyla (97) as well as by diminished microbial richness and diversity (19, 32, 96, 101–103). Low microbial gene richness was associated with more adiposity, dyslipidemia and insulin resistance (101). In the same vein, diminished microbial gene richness has been related to low-grade inflammation and marked dysmetabolism (102) and, even among individuals with severe obesity, those patients with lower microbial gene richness had worse metabolic conditions (104). Among microbial genes associated with obesity, 75% belongs to *Actinobacteria* and 25% to *Firmicutes*, whereas 42% of lean-associated microbial genes belong to *Bacteroidetes* (96, 105, 106).

Gut microbiota composition from diabetic patients also differs from healthy subjects (107–109). Munukka et al., analyzed gut microbiota from women with and without metabolic disorders and concluded that the differences in some bacteria belonging to *Eubacterium rectal-Clostridium coccoides* group were more associated with obesity-related metabolic disorders than with obesity *per se* (110). Evidence also suggest that the amount of specific gut bacterial groups might also influence lipid metabolism. To be more precise, *Lachnospiraceae* was related to low LDL-C levels and both *Pasteurellaceae* and *Collinsella* associated with low triglyceride levels. *Tenericutes* and *Butyricimonas* were also related to a favorable lipid profile with low triglyceride and high HDL-C levels (111). Therefore, gut microbiota has been related to the different hallmarks of the metabolic syndrome.

These data point out the gut microbiota as a crucial player in the regulation of host's energy homeostasis, though the mechanism by which the different bacterial species contribute to the regulation of metabolic functions are not fully understood. Current evidence point at low-grade inflammation and microbiota-derived metabolites as the possible links in the interplay of the gut microbiota and host's metabolism. These topics have been extensively reviewed elsewhere (19–21, 112) and will only be briefly mentioned in this review.

As above mentioned, low-grade inflammation is a hallmark of obesity and its related metabolic complications. Inflammatory mediators interfere insulin signaling promoting insulin resistance. Immune cell infiltration in metabolic tissues impair its function and is enhanced in metabolic diseases. Concordantly, inflammation is related to diabetes, unhealthy lipid profile and favors the development of atherosclerotic lesions (113). However, the precise factors triggering the inflammatory response in metabolic diseases remains unclear. Within this context, it has been hypothesized that bacterial products, such as the Gram-negative bacteria cell wall component, lipopolysaccharide (LPS), can translocate from the gut to the circulation (114). LPS translocation would be enhanced by the consumption of fatty meals as these molecules are packed into chylomicrons in the enterocytes and delivered into the circulation (115). In addition, a "leaky" gut due to compromised gut barrier integrity would also favor LPS translocation (114). Circulating gut-derived LPS, that activate the inflammatory response by binding to its receptor TLR-4, can reach metabolic tissues such as adipose tissue and induce inflammation, insulin resistance and impaired lipid accumulation (116). The inability of adipose tissue to store excess energy results in increased blood lipids and toxic lipid accumulation into non-fatty organs such as liver or muscle (117, 118). In agreement with this hypothesis, circulating LPS levels are closely related to triglyceride levels and blood pressure, augment upon high-fat meal intake (115, 116, 119, 120) and its increase is associated with inflammation and lower expression of lipogenic markers in the adipose tissue of obese patients, as recently confirmed by our group (116).

During the fermentation of indigestible dietary substrates, intestinal bacteria produces different by-products such as short-chain fatty acids (SCFAs). SCFAs, such as butyrate, acetate and propionate, take part in a variety of physiological functions. SCFAs have been shown to regulate adiposity, to improve insulin

sensitivity, to exert anti-inflammatory action by modulating immune cells and to regulate incretin secretion. In addition, these metabolites also participate in the maintenance of the gut barrier integrity, decreasing the risk of bacterial product translocation from the gut to the circulation (19, 21, 121–123). Butyrate represents a relevant energy source for colonocytes, contributes to epithelial cell health and consequently, to the integrity of intestinal epithelia. In addition, anti-inflammatory properties have been attributed to this SCFA (20, 124). Propionate stimulates the release of the satiety-inducing incretins GLP-1 and peptide YY, resulting in reduced food intake and concomitant weight, visceral adipose tissue and hepatic fat reduction, and preserved insulin sensitivity (125). Acetate is used by adipocytes, muscle or liver as energy substrate. It has been suggested that elevated levels of acetate and propionate associates with satiety, weight loss, and decreased inflammatory markers and blood lipids (126). However, some controversial results indicate that, when highly produced by gut microbiota, acetate could activates several pathways stimulating insulin secretion, hyperphagia and obesity in animal models (127). Then, the harmful or beneficial effects of SCFAs might be dependent on their levels as well as on the interaction with other factors (20). Notwithstanding this, human studies showed that obese and diabetic patients had diminished SCFA-producing bacteria and SCFAs (128). Therefore, SCFAs can mediate the multiple effect that gut microbiota exert on host´s metabolism.

Modulation of bile acid dynamics by gut microbiota has been also related to metabolic regulation (21). Bile acids participates in fat emulsification and absorption of lipids and liposoluble vitamins in the intestine. Primary bile acids are produced by hepatocytes, released in the proximal intestine, reabsorbed in distal ileum and recycled by the liver. Besides facilitating fat digestion and absorption, bile acids exerts metabolic functions by means of their receptors farnesoid X receptor (FXR) and Takeda G- protein receptor-5 (TGR5). FXR and TGR5 signaling promotes hepatic glycogen synthesis and insulin sensitivity, insulin secretion, energy expenditure in liver brown adipose tissue and muscle, thermogenesis and satiety (76). Gut microbiota metabolizes and deconjugates primary bile acids to transform them into secondary bile acids (129). Therefore, gut microbiota play a relevant role in determining the composition of bile acid pool which is relevant to the final biological actions of these molecules (130). In fact, it has been suggested that secondary bile acids can differentially interact with FXR and TGR5 than primary bile acid which would result in distinct metabolic actions (112, 131).

Obviously, bacterium by-products generated by the gut microbiota is highly determined by the diet. Bacteria are highly specific on the kind of nutrient they metabolize. Then, the effect of a certain type of diet on adiposity and metabolism activation not only is determined by calorie amount or energy harvesting by gut bacteria, but it is also defined by the specific kind of nutrient that enhances the growth of specific bacterial species damaging to coexisting microorganism by bacterial competition (99). For instance, some gut bacterial groups metabolized choline and L-Carnitine from dietary source (e.g. red meat or eggs) into trimethylamine (TMA). TMA is absorbed and oxidized by hepatic flavin monooxygenase 3 (FMO3) to produce trimethylamine *N*-oxide (TMAO) (132). Elevated levels of this metabolite have been described in diabetic (132) and obese (133) individuals and correlates with cardiovascular disease (134).

The production of these metabolites, the integrity of gut mucosa and consequently the actions that gut bacteria would exert as a whole, would depend on the abundance and proportion of each bacterial taxa inhabiting our intestine. Determinants of gut microbiota composition include dietary intake or antibiotic treatment, among other exogenous and endogenous factors (20, 30, 135, 136). Disruption of the gut bacterial equilibrium results in the deregulation of host metabolic functions and triggers the onset of obesity and obesity-related metabolic diseases (100, 112).

In this line, several studies aimed at assessing whether the modulation of the gut microbiota by antibiotic administration was related to obesity and other metabolic variables (31–34, 137, 138). Associations of diverse antibiotic treatments in early life with increased risk of childhood obesity has been reported (33, 34). A recent study focused on analyzing the gut microbiota profiles associated with the increased risk of childhood obesity due to early antibiotic exposure. This study showed that boys were at higher risk of increased abdominal adiposity than girls exposed to several courses of antibiotics and that changes in specific bacterial groups were related to both repeated antibiotic exposure and childhood adiposity (31). By contrast, Ajslev et al., described that, when mothers were overweight, early antibiotic exposure were related to decreased risk of developing childhood obesity. Unfortunately, gut microbiota was not analyzed in this study so it cannot be determined whether specific modifications in gut bacterial groups could account for the differential risk of developing obesity depending on maternal weight (137).

In the adult population, the association between antibiotic treatment, gut microbiota and insulin sensitivity is still controversial (32, 138). Vrieze et al., found that 1-week vancomycin treatment increased and decreased primary and secondary bile acids, respectively, and decreased insulin sensitivity evaluated 2-3 days after treatment cessation. However, amoxicillin administration had no effects on the study variables (32). Despite significant changes in gut microbiota diversity and composition as well as in SCFA and bile acids levels, Reijnders et al., did not find significant effects after 1-week vancomycin or amoxicillin exposure on different markers of insulin sensitivity, metabolic, hormonal or inflammatory parameters evaluated 8-weeks after treatment (138).

These findings point out the fact that factors such as age, gender, previous obesity degree, the type of antibiotic or post-therapy evaluation time could influence the effects on host's metabolism. Therefore, further studies are required to elucidate how specific gut microbiota modulation exerted by each kind of antibiotic therapy affects metabolic homeostasis according to host's characteristics. In view of the multiple therapeutic options for *H. pylori* eradication, this perspective become particularly relevant in the management of *H. pylori* infection. In addition, it is of interest to elucidate whether gut microbiota modulation by antibiotic eradication therapy improves or worsens the previous impaired metabolic status of *H. pylori* patients.

GUT MICROBIOTA IN *H. PYLORI* INFECTION

H. pylori colonizes gastric mucosa leading to modification in the gastric microenvironment and disturbing gastric microbiota composition (139). These changes has serious local effects on stomach, but it also affects the function of other regions of the gastrointestinal tract which likely results in altered absorption of nutrients and drugs as well as on the production of incretins involved in metabolic homeostasis. Furthermore, few years ago, it was also proposed that *H. pylori* infection might also trigger large intestinal microbiota changes (140). Although close attention has been paid to the effect of *H. pylori* eradication treatment on gut microbiota, very recent human studies have also confirmed that *H. pylori* infection *per se* is linked to gut dysbiosis including alterations in bacterial diversity and abundance (9–18). A summary of bacterial shifts reported in individuals infected by *H. pylori* is displayed in **Table 1**.

Within this context, our group and others have reported that human *H. pylori* infection is related to changes in bacterial diversity, but findings still controversial (10–14, 16). Some studies observed less diversity in *H. pylori* positive than in *H. pylori* negative subjects (10, 11). In view of the fact that *H. pylori* infection is usually related to unhealthy profile, these results are congruent with the general idea that high gut microbial diversity is an indicator of a healthy gut microbiome (101). In fact, low bacterial richness has been related to insulin resistance, dyslipidemia and higher overall adiposity (32). Nevertheless, other authors intriguingly found that several alpha diversity estimators exhibited higher scores, which indicate higher level of gut microbiota complexity in *H. pylori* cases (12–14, 16). Different study population, calculation of different diversity indexes or techniques for quantifying gut microbiota might underlie this opposite findings. In addition, other factors such diet or disease severity could be also influencing gut diversity. Therefore, controlled trials with homogenous designs are required to elucidate the actual effect of *H. pylori* infection and diversity of gut microbiota.

Regarding the specific groups of gut bacteria, some human studies have shown that the relative abundances of dominant phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* significantly differ in the gut of *H. pylori* positive individuals compared to negative controls, although trends towards the increase, decrease or no differences have been reported (10, 14–16). Similarly, some studies found that *H. pylori* infection decreases the abundance of *Actinobacteria* (10, 11), but He et al., reported an increase in this Phylum (16). Similar to bacterial diversity, variability in study population including age or geographical region and different exclusion and inclusion criteria might underlie the heterogeneity in major phyla proportion in *H. pylori* infected patients. In fact, it has been reported that gut microbiota can differ between Japanese and other populations (142). Differences in these phyla have been also related to obesity and Type 2 Diabetes (19). Microbial pattern with low *Bacteroidetes* and high *Actinobacteria* and *Firmicutes* proportions characterized the gut microbiota from obese patients (19, 96, 103). Therefore, further studies on *H. pylori* positive patients with more homogenous population might be helpful to establish consistent trends among predominant phyla in relation to metabolic diseases.

Despite not having found consistent results in the main phyla related to *H. pylori* infection, in general terms and taking into account the diversity of bacterial functions included in each phylum, bacterial changes at the level of the different taxonomic categories belonging to each phylum may have both positive and negative effects on the host. In this line, Frost et al., found that *H. pylori* infected individuals displayed elevated levels of the facultative pathogen *Haemophilus* but decreased levels of *Pseudoflavonifractor* and *Parasutterella* (13). *Pseudoflavonifractor* encompasses butyrate-producing bacteria and *Parasutterella* are succinate-producing bacteria (143). Thus, the decrease of *Pseudoflavonifractor* might partly explain the negative impact of *H. pylori* infection on metabolic profile as the result of reduced SCFA production. Notwithstanding this, the genus *Parasutterella* is increased in Crohn's disease (144), so authors hypothesized that *H. pylori* might be exerting a protective role against pathogens related to other gastrointestinal diseases (13). In fact, succinate has been described as a virulence factor that might exacerbate enteric infections (145). In addition, this bacterium together with other taxa increased by *H. pylori* (e.g. *Alistipes*), have been proposed as potential predictor biomarkers of obesity-related metabolic abnormalities (146). In the same vein, succinate-producing bacteria and elevated levels of succinate have also been associated with Type 2 Diabetes and obesity (147).

By using shotgun metagenomic sequencing, Wang et al., found variation in other bacterial species according to the presence or absence of *H. pylori* infection (141). They specifically found increased proportions of *Prevotella copri*, a proinflammatory bacterium (148) as well as *Klebsiella pneumoniae* and *Enterobacter cloacae*, two infectious bacteria (149). These results suggest that *H. pylori* infection may promote growth of harmful bacteria in the gut. Conversely, *Sutterella wadsworthensis*, *B. vulgatus*, and *E. coli* amounts were lower in the *H. pylori*-positive compared to non-infected individuals (141). Members of the genus *Sutterella* are highly prevalent commensals with the ability to adhere to intestinal epithelial cells indicating a possible immunomodulatory role (150). In addition, the pathobiont *Bacteroides vulgatus* has been implicated in the etiology of both Crohn's disease and ulcerative colitis, but little is known about how its functional activity might drive the host inflammatory response (151).

A study carried out in a Japanese population specifically focused on analyzing how *H. pylori* modulates the proportion of each *Lactobacillus* species in the gut and found higher relative abundance of *Lactobacillus* in *H. pylori* infected patients than in non-infected individuals (18). *Lactobacilli* endows gastrointestinal tract with "colonization resistance" serving as a defense mechanism which protects the host from potentially pathogenic microbials. Probiotic effects have widely been attributed to different *Lactobacillus* species such as *L. casei* or *L. rhamnosus* that prevented antibiotic-related diarrhea (152), which may be due to the role of these bacteria in gut barrier preservation (153). Furthermore, certain *Lactobacillus* species have been also related to positive metabolic outcomes. While *Lactobacilli* relative abundance in the gut microbiota in obesity and after weight loss is controversial, it has been described that specific *Lactobacillus* strains (e.g. *L. rhamnosus*, *L. gasseri*, *L. plantarum* or *L. paracasei*) relates with a reduction of obesity-associated metabolic disorders and even an

TABLE 1 | Human studies analyzing the impact of *H. pylori* infection on the gut microbiota determined by bacterial DNA sequencing.

Study (Reference)	Design	Methodology	Shifts in gut bacterial groups in HP+
Benavides-Ward et al. (15)	Paediatric Asymptomatic Peruvian population (Age=6-12 y). Study groups: HP+ (n=28) *vs* non-infected (n=28) children	Targeted sequencing	↑*Proteobacteria*, ↑*Firmicutes*, ↑*Clostridium*, ↑*Prevotella*
Chen et al. (17)	Adult Chinese population (age = 18-70 y). Study groups: HP+ (n=70) vs. *H. pylori*-negative (n=35) subjects	16S rRNA V3-V4 region sequencing (MiSeq Platform)	↑*Sphingomonas* sp., ↑*Turicibacter* sp. ↓*Nitrospirae* ↓*Bacteroides plebeius*
Dash et al. (12)	Pooled Young and Adult Arabic population (Age=15.5-59 y). Study groups: Asymptomatic HP+ (n=12) *vs. H. pylori*-negative (n=48) subjects	16S rRNA V4 region sequencing (MiSeq Platform)	↑Diversity ↑*Succinivibrio*, ↑*Turicibacter* ↑*Coriobacteriaceae*, ↑*Rikenellaceae* ↑*Desulfovibrio*, ↑*Enterococcaceae*
Frost et al. (13)	Adult Caucasian population (SHIP cohorts; Age= 43-63 y). Study groups: HP+ (n=212) *vs. H. pylori*-negative (n=212) subjects	16S rRNA V1-V2 region sequencing (MiSeq Platform)	↑ Diversity ↑ *Haemophilus* ↓*Parasutterella*, ↓*Pseudoflavonifractor*
Gao et al. (14)	Adult Chinese population (Age=40-69 y). Study groups: HP+ (n=24) vs. *H. pylori*-negative (n=15) subjects	16S rRNA V4 region sequencing (MiSeq Platform)	↑Diversity ↑*Bacteroidetes*, ↑*Firmicutes*, ↑*Proteobacteria*
He et al. (16)	Young Chinese population (Age=21-30 y). Study groups: Asymptomatic HP+ (n=17) *vs. H. pylori*-negative (n=7) subjects	16S rRNA V3-V4 region sequencing (MiSeq Platform)	↑Diversity ↑*Proteobacteria*, ↑*Actinobacteria*, ↑*Acidobacteria*, ↑*Alistipes* ↓*Subdoligranulum*, ↓*Lachnoclostridium*
Iino et al. (18)	Adult Japanese population. Study groups: HP+ (n= 226) vs. *H. pylori*-negative (n=524) subjects	16S rRNA V3-V4 region sequencing (MiSeq Platform)	↑*L. salivarius*, ↓*L. acidophilus*
Martin-Nunez et al. (10)	Adult Caucasian population (Age=18-65 y). Study groups: HP+ (n=40) *vs. H. pylori*-negative (n=20) subjects.	16S rRNA V3-V4 region sequencing (MiSeq Platform)	↓Diversity ↑*Bacteroidetes*, ↓*Firmicutes* ↓*Actinobacteria* ↑*Bacteroidetes/Firmicutes* ratio
Wang et al. (141)	Adult Chinese population (Age=20-66 y). Study groups: HP+ (n=128) *vs. H. pylori*-negative (n=185) subjects.	Shotgun metagenomic sequencing (BGISEQ-500 platform)	↑*Prevotella copri* ↑*Enterobacter cloacae* ↑*Klebsiella pneumoniae* ↓*Sutterella wadsworthensis* ↓*B. vulgatus* ↓*E. coli*
Wu et al. (11)	Adult Chinese population (Age=18-65 y). Study population: HP+ (n= 40) *vs. H. pylori*-negative (n=20) subjects	16S rRNA V4 region sequencing (MiSeq Platform)	↓Diversity ↓*Actinobacteria*, ↓*Gemmatimonadetes*, ↓*Nitrospirae*, ↓*Chlorobi*, ↓*Thermi*, *WS3*, ↓*Caldithrix*.

The most significant bacterial groups showing differential proportions in H. pylori patients compared to non-infected patients are shown. HP+, H. pylori positive patients.

improvement on insulin sensitivity. However, other species such as *L. reuteri* has positively been associated with adiposity, which suggests *Lactobacillus* strain-dependent physiological effects on metabolism and weight regulation (19, 154–156). Iino et al., found that *H. pylori* positive patients displayed reduced amounts of *L. acidophilus* and increased proportion of *L. salivarius* in comparison with non-infected subjects. Authors suggested that alterations in *Lactobacillus* proportions could be related to the suppression of gastric acid secretion by *H. pylori* infection, but putative physiological effects that these changes can have on host's metabolism and health have not yet been addressed (18).

In the same way that gastric colonization by *H. pylori* can affect the composition of gut microbiota, some gut bacteria might be also influencing the bacterial colonization of other gastrointestinal regions, including *H. pylori* in the stomach. In this regard, Chen et al., found that, despite not having noticing significant differences in the abundance of several putative beneficial taxa including *Bifidobacterium*, *Lactobacillus*, *Clostridium butyricum*, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, the phylum *Nitrospirae* exclusively appeared in *H. pylori*-negative subjects (17). Similarly, in the study by Wu et al., this phylum presented low values in patients with duodenal ulcer and *H. pylori* infection (11). *Nitrospirae* are the most abundant and diverse nitrite-oxidizing bacteria which convert nitrite to nitrate (157). Of note, nitrite was shown to have bactericidal effects against *H. pylori* (158).

Differences between the gut microbiota from asymptomatic *H. pylori* infected patients *vs.* non-infected subjects have been also reported (12, 15, 16). In fact, *H. pylori* infection alters the gut microbiota in this asymptomatic patients by increasing *Proteobacteria*, *Clostridium*, *Firmicutes* and *Prevotella* in a paediatric population (15) and members belonging to *Succinivibrio*, *Coriobacteriaceae*, *Enterococcaceae*, and *Rikenellaceae* in adults (12) compared to non-

infected subjects. The study by He et al., identified their study participants as asymptomatic although they also stated that subjects were diagnosed with superficial gastritis (16). To the best of our knowledge, it has not yet been evaluated whether the gut microbiota from asymptomatic *H. pylori*-infected patients also differs from that from symptomatic *H. pylori*-infected patients. This information could be of great interest to shed light on the possible role of the gut microbiota composition as player and/or biomarker of the severity of *H. pylori* infection which ranges from asymptomatic to serious clinical manifestation such as gastric cancers.

In this line of thought, Gao et al. investigated the association between the gut microbiota and *H. pylori*-related gastric lesions. To this end, they compared the intestinal microbiota of subjects without gastric injury, with gastritis or with intestinal metaplasia, and concluded that indeed the alterations of the fecal microbiota, particularly the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, are likely involved in the process of progression of the gastric disease associated with *H. pylori* (14).

In addition, Frost et al., found that there were bacterial significant variations even within the *H. pylori* positive patient group that was dependent on the individual *H. pylori* antigen load. To be more precise, a negative association was reported between antigen load and *Bacteroides*, *Fusicatenibacter*, *Barnesiella*, and *Alistipes*, taxa exhibiting putative healthy characteristics at first sight. *Fusicatenibacter* and *Alistipes* are butyrate and lactate producing bacteria that could exert anti-inflammatory and other beneficial actions (159, 160). However, opposite results regarding different species belonging to *Alistipes* taxa and its relationship with dysfunction of several organs such as liver or cardiovascular system has been described (161). In fact, He et al., who also found that *H. pylori* infected patients had elevated levels of *Alistipes* compared to non-infected patients, referred to an animal study which found that this taxa was positively correlated with weight, fat mass, LPS and inflammatory cytokines (16, 162). On the other hand, *Barnesiella* is associated with more efficient eradication of antibiotic-resistant bacteria (163). By contrast, authors stated that non-significant results were retrieved from analyses taking into account *H. pylori* serology level (13). In the same vein, Iino et al., found that the relative abundance of *Lactobacillus* was significantly higher in *H. pylori* positive patients with severe atrophic gastritis as compared with infected patients with mild atrophy gastritis or without gastritis (18) supporting the hypothesis that gut microbiota could also differ depending on symptoms severity. Of note, no differences according to the degree of atrophic gastritis was found in the specific *Lactobacillus* species studied (*L. acidophilus* and *L. salivarius*) (18) which remarks the necessity to find the specific bacterial groups that can be related to infectious severity.

MODULATION OF THE GUT MICROBIOTA BY ANTIBIOTIC-BASED *H. PYLORI* ERADICATION THERAPIES

According to the Kyoto consensus report, *H. pylori* gastritis has to be regarded as an infectious disease and the resulting recommendation is the treatment of all *H. pylori* infected patients regardless the clinical manifestations (164). Antibiotic therapies are the first-line treatment for *H. pylori* eradication, but the increasing antibiotic resistance rates make necessary the adaptation of the treatment regimen. In general terms, the standard triple therapy based on the combination of one proton pump inhibitor (PPI), clarithromycin and amoxicillin or metronidazole is recommended when *H. pylori* clarithromycin resistance is low (<15%). By contrast, quadruple therapy based on bismuth administration together two antibiotics and one PPI is the first-line treatment for *H. pylori* eradication in regions with high antibiotic resistance rates (27, 28). However, recent reports highlight how the increasing resistance to antibiotics compromises the efficacy of these recommended therapies and salvage regimens have to be used (28, 29). This lead to high variability on the type and amount of specific antibiotics that can have different impact on the patient's health.

Antibiotic *H. pylori* therapies are not exempt from side effects. The extensively reported gastrointestinal adverse events associated to antibiotic administration such as nausea, vomiting or diarrhea among others, are linked to quantitative and qualitative changes in the gut microbiota (165). Antibiotic-induced changes in the gut microbiota has become a matter of concern in the treatment of *H. pylori* as the essential role of intestinal-residing bacteria in maintaining human health beyond gut function is increasingly accepted (27).

There is growing evidence that antibiotic-based therapies for *H. pylori* eradication promote alterations in the gut microbiota (10, 11, 16, 17, 24, 35–44), but different antibiotic combinations can exert differential effects on microbial community (166) (**Table 2**). In addition, proton pump inhibitors (PPI) which is usually administered together antibiotics for *H. pylori* eradication also contributes to bacterial shifts in the intestine (167).

In general terms, antibiotic administration has been related to a decrease in bacterial diversity (30, 138). Regarding the various *H. pylori* eradication therapies it has been reported a decrease in bacterial diversity after triple therapy consisting of PPI, amoxicillin and clarithromycin for 7 days (39, 40, 43), 10 days (10) or 14 days (11, 36). Nevertheless, most of these studies found that bacterial diversity was restored in the short and the long-term after treatment cessation (11, 36, 40, 43). However, other studies did not reported an improvement of bacterial diversity after cessation of triple therapy (10, 39).

Quadruple therapy with bismuth during 10 or 14 days also led to decreased bacterial diversity but not all the studies found a total recovery of bacterial diversity evaluated from 6 weeks to 1 year post-treatment (16, 36, 38, 44). By contrast, concomitant therapy of three antibiotics (amoxicillin, clarithromycin, metronidazole) without bismuth for 14 days decreased bacterial diversity, but it was fully (35) or partly restored (36) at 2 months or 1 year post-treatment, respectively.

Liou et al., compared the effect of triple, quadruple and concomitant therapies on bacterial diversity and found that the two latter treatments were related to lower alpha diversity in comparison with triple therapy at 1 year post-eradication treatment (36).

Notably, few studies have contrasted eradication therapy-induced bacterial diversity changes with bacterial diversity in the gut of non-infected patients (controls) which would drop hint at the similarity of bacterial diversity scores before and after

TABLE 2 | Effect of antibiotic *H. pylori* eradication therapies on gut microbiota composition in human studies.

Studies (Reference)	Design	Eradication therapy	Evaluation post-therapy	Changes in the gut microbiota after eradication therapy	Methodology
Jakobsson et al. (40)	Adult European population (Age =50-75 y). Comparison groups: HP+ *vs* post- eradication therapy (n=3).	PPI, amoxicillin, clarithromycin for 7 days.	8-13 days	↓ Alpha diversity, ↓*Actinobacteria*, ↑*Enterococcus*, ↓*Lachnospiraceae*, ↓*Clostridia*, ↓*Bifidobacteria*.	16S rRNA (454-based pyrosequencing) and TRFLP
			1 - 4 years	Alpha diversity restored, but microbiota composition NO returned to baseline.	
Oh et al. (41)	Adult Asian population (Average age = 49.3 y). Comparison groups: HP+ *vs* post- eradication therapy (n=10).	PPI, Clarithromycin, Amoxicillin for 14 days.	2 weeks	↓*Bacteroidetes*, ↓*Firmicutes*, ↓*Prevotella copri*, ↑*Proteobacteria*.	16S rRNA (V1-V3). Roche/454 GS Junior platform
Yap et al. (42)	Young adult Asian population (Age= 18-30 y). Comparison groups: HP+ *vs* post- eradication therapy (n=17).	PPI, amoxicillin, clarithromycin for 7 days.	6 months	↓*Actinobacteria*, ↓*Proteobacteria* ↑*Verrucomicrobia*	16S rRNA (V3-V4). Miseq platform (Illumina)
			6 and 12 months	↑ *Firmicutes*, ↓*Bacteroidetes*	
			12 and 18 months	↑*Proteobacteria*, ↑*Actinobacteria*, ↑*Verrucomicrobia*.	
Yanagi et al. (24)	Adult Asian population (Age= 42-80 y). Comparison groups: HP+ *vs* post- eradication therapy (n=20).	PPI, amoxicillin, clarithromycin for 7 days.	1 week	↑*Bacteroidetes*, ↑Archaea, ↓*Actinobacteria*, ↓*Proteobacteria*.	16S rRNA. Miseq platform (Illumina),
			3 months	↑ B:F ratio, ↓*Actinobacteria*, ↓*Bifidobacterium*, ↑*Faecallibacterium prausnitzii*.	
Chen et al. (17)	Adult Asian population (Age= 18-70 y). Comparison groups: HP+ *vs* post- eradication therapy (n=35).	PPI, amoxicillin, furazolidone, colloidal bismuth pectin for 14 days.	14 days	↓*Firmicutes*,↓*Bacteroidetes*,↓*Verrucomicrobia* ↓*Lentisphaerae*, ↓B:F ratio, ↓*Lachnospiraceae*, ↓*Ruminococcaceae*, ↑*Proteobacteria*, ↑*Cyanobacteria* ↑*Klebsiella*.	16S rRNA (V3-V4). Miseq platform (Illumina)
			14 and 56 days	↑Enterobacteriaceae, ↑Leuconostocaceae, ↓Rikenellaceae, ↓Christensenellaceae, ↓Peptococcaceae, ↓Clostridiales Family XI, ↓Victivallaceae	
Gotoda et al. (43)	Adolescent Asian population (Age=14-15 y). Comparison groups: HP+ *vs* post- eradication therapy (n=8).	PPI, amoxicillin, clarithromycin for 7 days.	1 week	↓Alpha diversity, ↓*Actinobacteria*, ↓*Bifidobacteriales*.	16S rRNA (V3-V4). Miseq platform (Illumina)
			2 months	Microbiota returned to baseline.	
Hsu et al. (44)	Adult Asian population (Average age=48.8 y). Comparison groups: HP+ *vs* post- eradication therapy (n=11).	PPI, bismuth, metronidazole, tetracycline for 14 days.	2 weeks	↓Alpha Diversity, ↓*Bacteroidetes*,↓*Actinobacteria*, ↓*Verrucomicrobia,* ↑*Proteobacteria,* ↑*Klebsiella,* ↑*Morganella*, ↑*Proteus,* ↑*Serratia*, ↑*Escherichia*, ↑*Cyanobacteria*, ↑*Enterococcus,* ↑*Streptococcus*	16S rRNA (V3-V4). Miseq platform (Illumina)
			8 and 48 weeks	Microbiota returned to baseline. Alpha diversity restored at 48 weeks.	
Hsu et al. (35)	Adult Asian population (Average age=53 y). Comparison groups: HP+ *vs* post- eradication therapy (n=12)	PPI, amoxicillin, clarithromycin, metronidazole for 14 days.	2 weeks	↓ Alpha diversity, ↓*Bacteroidetes,* ↓*Firmicutes*, ↓*Actinobacteria,* ↑*Proteobacteria*, ↑*Escherichia,* ↑*Proteus,* ↑*Salmonella,* ↑*Klebsiella,* ↑*Morganella*.	16S rRNA (V3-V4). Miseq platform (Illumina)
			8 and 48 weeks	Microbiota composition and Alpha diversity returned to baseline.	
He et al. (16)	Asymptomatic Young Asian population (Age=21-30 y). Comparison groups: **a)** HP+ *vs* post- eradication therapy (n=10).	PPI, bismuth, amoxicillin, furazolidone for 14 days.	6 weeks	**a)** ↓Alpha Diversity. No significant differences in phyla. **b)** No significant differences in Alpha diversity	16S rRNA (V3-V4) Miseq platform (Illumina)
			26 weeks	**a)** ↓Alpha Diversity, ↓*Proteobacteria,* ↓*Bacteroidetes*, ↓*Alistipes*, ↑*Firmicutes,* ↑*Lachnoclostridium*, ↑*Blautia*. **b)** Gut microbiota acquired a negative control-like profile.	

(Continued)

TABLE 2 | Continued

Studies (Reference)	Design	Eradication therapy	Evaluation post-therapy	Changes in the gut microbiota after eradication therapy	Methodology
	b) Post- eradication therapy (n=10) *vs* controls (n=7).				
Liou et al. (36)	Adult Asian population (Age>20 y). Comparison groups: HP+ *vs* post- eradication therapy (n=408).	PPI, amoxicillin, clarithromycin for 14 days.	2 weeks	↓Alpha diversity, ↓*Fusobacteria*	16S rRNA (V3-V4). Miseq platform (Illumina)
			8 weeks and 1 year	Alpha diversity returned to baseline Microbiota returned to baseline.	
	Adult Asian population (Age>20 y). Comparison groups: HP+ *vs* post- eradication therapy (n=410)	PPI, amoxicillin, clarithromycin, Metronidazole for 10 days.	2 weeks	↓Alpha diversity, ↓Beta diversity, ↓*Bacteroidetes*, ↓*Firmicutes*, ↓*Fusobacteria*, ↑*Proteobacteria*.	
			8 week and 1 year	↓Alpha diversity, Beta diversity restored at year 1. Microbiota returned to baseline.	
	Adult Asian population (Age>20 y). Comparison groups: HP+ *vs* post- eradication therapy (n=396).	PPI, bismuth, metronidazole, Tetracycline for 10 days.	2 weeks	↓Alpha diversity, ↓Beta diversity, ↓*Bacteroidetes*, ↓*Firmicutes*, ↓ *Fusobacteria*, ↑*Proteobacteria*.	
			8 weeks and 1 year	↓Alpha diversity, ↓Beta diversity. Microbiota returned to baseline.	
Martin-Nunez et al. (10)	Adult European population (Age=18-65 y). Comparison groups: **a)** HP+ *vs* post- eradication therapy (n=40). **b)** Post- eradication therapy (n=40) *vs* controls (n=20).	PPI, clarithromycin, amoxicillin for 10 days.	2 months	**a**)↓ Richness, ↓*Actinobacteria*, ↓*Bifidobacteriaceae*, ↓*Bifidobacterium*, ↓*B. longum*, ↓*B. adolescentis*, ↓*Streptococcaceae*, ↓*Streptococcus* **b**) ↓Alpha diversity, ↑*Bacteroidetes*, ↓*Firmicutes*, ↓ *Actinobacteria*, ↓*Turicibacter*, ↓*Ruminococcaceae*, ↓Oscillospira, ↓*Oxalobacteriaceae*, ↓*Oxalobacter*, ↓*O. Formigenes*, ↓*Enterobacteriaceae*.	16S rRNA Miseq platform (Illumina)
Olekhnovich et al. (37)	Adult European population (Average age= 47.7 y). Comparison groups: HP+ *vs* post- eradication therapy (n=40).	PPI, bismuth, amoxicillin, clarithromycin + prebiotic for 14 days.	16 days	↓Alpha Diversity, ↓ *Actinobacteria*, ↓*Bifidobacterium adolescentis*, ↓*Verrucomicrobia*, ↑*Enterococcus faecium*, ↓Eubacteriaceae, ↓*Lachnospiraceae*, ↓*Ruminococcaceae*	Whole genome shotgun sequencing on ABI/SOLiD 5500W platform (Life Technologies)
Wu et al. (11)	Adult Asian population (Age= 18-65 y). Comparison groups: HP+ *vs* post- eradication therapy (n=20)	PPI, clarithromycin and amoxicillin for 14 days.	2 weeks	↓Alpha diversity, ↓*Tenericutes*	16S rRNA. Miseq platform (Illumina)
			4 weeks	↑*Bacteroidetes*	
			8 weeks	Microbiota returned to baseline.	
Kakiuchi et al. (39)	Adolescent Asian population (Age=15 y). Comparison groups: HP+ *vs* post- eradication therapy (n=31)	PPI, amoxicillin, clarithromycin for 7 days.	Therapy day 7 8-12 weeks after treatment	↓Alpha diversity, ↓*Collinsella*, ↓*Bifidobacterium.*	16S rRNA gene (V3-V4). Miseq platform (Illumina)
Tang et al. (38)	Adult Asian population (Age=18-65 y). Comparison groups: HP+ *vs* post- eradication therapy (n=74).	PPI, amoxicillin, furazolidone, bismuth potassium citrate for 14 days.	2 weeks	↓ Alpha diversity, ↑*Proteobacteria*, ↓*Firmicutes*, ↓*Bacteroidetes*, ↓ *B:F,* ↑*Shigella,* ↑*Klebsiella,* ↑*Streptococcus,* ↑*Veillonella*, ↓*Bacteroides*, ↓*Faecalibacterium*, ↓*Roseburia*, ↓*Phascolarctobacterium*, ↓*Blautia*	16S rRNA gene (V3-V4). Miseq platform (Illumina)
			4 weeks	↓ Alpha diversity	
			6 and 8 weeks	Alpha diversity restored. No significant differences in phyla.	

The most significant and consistent changes in gut microbiota after the various H. pylori eradication therapies an studies are summarized in this table. B:F, Bacteroidetes/Firmicutes ratio; PPI, proton pump inhibitor; TRFLP, terminal-restriction fragment length polymorphism.

eradication treatment to those seen in healthy conditions. However, opposite evidence has been found with no differences in the gut bacterial diversity at 6 and 26 weeks post-treatment with quadruple therapy compared to controls (16) or lower bacterial diversity evaluated at 2 months after triple therapy than in both controls and *H. pylori* infected patients before undergoing eradication intervention (10).

Pooled together, these findings suggest that the effect on gut bacterial diversity and its length are dependent on the eradication therapy used. A recent meta-analysis stated that alpha diversity was reduced immediately within the first week after eradication, but no consistent conclusions were drawn from studies evaluating bacterial diversity at longer evaluation times (168). It is worth of mention that this meta-analysis pooled different kinds of eradication therapies to analyze the effect on bacterial diversity, which could limiting the conclusions by adding variability to the results. This meta-analysis conclude that further studies will be required to gain more evidence before raising firm conclusions.

The effect of the diverse treatments used for *H. pylori* eradication has been also assessed in terms of bacterial abundance. Many studies have reported changes in specific bacterial taxa, but most of these findings remains controversial (10, 11, 16, 17, 24, 35–44). While some studies have observed a decrease in *Firmicutes*, *Bacteroidetes*, and an increase in *Proteobacteria* 2 weeks after triple (41), quadruple (17, 36, 38, 44) or concomitant (35) therapies, other studies have shown an increase in *Bacteroidetes* (11) and a decrease in *Proteobacteria* (11, 24) after triple therapy. The phylum *Actinobacteria* (24, 40, 43, 44) and members belonging to this phylum as *Bifidobacteriales* (43), *Bifidobacterium* (39), *Bifidobacteria* (40), and *Bifidobacterium adolescentis* (37) also remained decreased at 1 or 2 weeks post-treatment regardless of the therapy used. *Bifidibacterium* are regarded as beneficial bacteria for host's health which promote gut barrier integrity, prevent gut mucosa colonization by opportunistic pathogens and are also involved in carbohydrate metabolism (169). SCFA producing bacteria such as *Lachnospiraceae*, *Ruminococcaceae*, *Eubacteriacea*, *Bacteroides*, *Faecalibacterium*, *Roseburia*, *Phascolarctobacterium* were also compromised at the short-term of the antibiotic treatment (**Table 2**). These bacteria might exert beneficial actions for the health as a consequence of the SCFA production. Concordantly, the decline of *Eubacteriaceae*, *Lachnospiraceae* and *Ruminococcaceae* has been associated with a broad spectrum of disorders (170).

On the contrary, the relative abundance of several putative detrimental bacteria which can release harmful factors for host's health, such as *Escherichia*, *Proteus*, *Morganella* (35, 44), *Serratia* (44), *Klebsiella* (17, 35, 38, 44) and *Streptococcus* (38, 44) augmented upon antibiotic administration (quadruple or concomitant therapy) (**Table 2**).

Most of the studies tested whether antibiotic-induced changes in the abundance of bacterial groups were restored upon treatment cessation. As summarized in **Table 2**, gut microbiota composition is restored in most cases at 2 months post-treatment. Nevertheless, it has been also reported that the imbalance of some bacterial groups remains in the short- and the long-term after treatment cessation (10, 16, 24, 40, 42). Jakobsson et al., documented changes in the gut microbiota that persisted for up to 4 years after *H. pylori* eradication, but formal statistical analysis was not done owing to small sample size (40). In the same way, other studies also reported persistent changes in gut microbiota i.e. a decrease in *Proteobacteria*, *Bacterioidetes*, *Actinobacteria* and an increase in *Firmicutes* at 6 (16, 42) and 12 moths post-triple therapy (42), as well as an enrichment in *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia* compared to baseline values at 18 months after treatment (42). Elevated relative *Bacteroidetes : Firmicutes* ratio was observed at 2 and 3 months of treatment cessation (10, 24). At phylum level, the meta-analysis made by Ye et al. showed a reduction in *Actinobacteria* and *Bacteroidetes* during the follow-up. In addition, *Firmicutes* was exclusively found to be augmented in the long-term after *H. pylori* eradication while *Poteobacteria* increased in short-term and returned to normality in the long-term (168). Lasting alterations in the proportions of these phyla may result in an altered production of bacterial metabolites which would affect host-bacterial crosstalk. For instance, acetate and propionate are mainly produced by *Bacteroidetes* members, while *Firmicutes* members typically produce butyrate (171). Notably, reduced *Bacteroidetes: Firmicutes* ratio and increased abundance of *Proteobacteria*, have been associated with obesity and the metabolic syndrome (19, 172).

Persistent antibiotic-induced shifts on bacterial groups can be associated with therapy adverse effects. In agreement with this, the relative abundance of the phylum *Proteobacteria* and some of its members including *Aggregatibacter* and *Sutterella* were higher at 2 weeks after quadruple therapy in patients who suffered from eradication intervention side effects compared with patients that did not reported adverse symptoms (44). *Proteobacteria* phylum includes many pathogens and it has been proposed that may be partly responsible for the development of adverse effects during eradication therapy (44).

Interestingly, some studies assessed the effect of probiotic supplementation administered together the antibiotic combination which attenuated the antibiotic-induced imbalance on gut microbiota composition (11, 41). Furthermore, probiotic supplementation have been associated with improved gastrointestinal symptoms (17, 39) and increased *Bacteroidetes: Firmicutes* ratio (17). All together, these effect might help to build up a beneficial gut microbiota profile after eradication therapy (38). This suggest that probiotic administration could attenuate antibiotic-induced gut dysbiosis, but to the best of our knowledge, it remains unexplored the consequences that this can have on host's metabolic health within the context of *H. pylori* infection.

The discrepant observations between studies could be due to different eradication regimens, drug doses, treatment duration and/or sample size. Furthermore, other factors such as dietary habits, resistance to antibiotics and differences in the rate of absorption of antibiotics can affect the influence of eradication therapy on the intestinal microbiota and the time required to restore original bacterial composition. On the other hand, it is remarkable that most of the studies did not include controls without *H. pylori* infection, and considered that the gut microbiota was restored when no significant differences were observed regarding baseline. In this sense, it should be taken into

account that pre-treatment bacterial composition is already influenced by *H. pylori* infection. This fact makes difficult to determine whether bacterial communities post-therapy mirror a healthy gut microbiota. All in all, further studies analyzing the effect of each eradication therapy and including non-infected controls, are necessary to clarify the effects that the various therapies for *H. pylori* eradication have on gut microbiota in the long-term and to elucidate the factors responsible for the variability in antibiotic response.

RELATIONSHIP BETWEEN METABOLIC TRAITS AND GUT MICROBIOTA MODIFICATIONS INDUCED BY *H. PYLORI* INFECTION AND THERAPY

As detailed above, evidence has shown that *H. pylori* infection as well as its eradication treatment lead to gut dysbiosis. However, despite the fact that gut microbiota is closely linked to metabolic health, the role of gut microbiota in the relationship between *H. pylori* infection and metabolic dysregulation has been scarcely studied. Several studies from our group, which analyzed gut microbiota from *H. pylori* infected patients before and after eradication treatment (antibiotic triple therapy based on omeprazole, clarithromycin, amoxicillin) as well as non-infected control patients, addressed this issue (**Table 3**) (10, 22, 23). It was found that *H. pylori* eradication treatment produces specific bacterial shifts associated with changes in glucose homeostasis-related parameters [HbA1c, glucose area under the curve (AUC) calculated upon an oral glucose tolerance test (OGTT) (10)], GLP-1 (23), ghrelin (24) and HDL-C levels (22).

To be more specific, changes in the amount of *Rikenellaceae*, *Butyricimonas*, *E. biforme*, *B. fragilis*, and *Megamonas* were inversely associated with changes in glucose levels or glucose-related parameters, i.e. HbA1c, in *H. pylori* subjects after eradication treatment (10). Several studies have shown that these bacteria are involved in the fermentation of non-digestible carbohydrates and the generation of SCFAs such as acetate, propionate and butyrate (173–175). In addition, *Rikenellaceae* and *Butyricimonas* members are also able to use the environmental glucose to produce SCFAs, which could contribute to glucose level regulation (175, 176).

It was not only analyzed glucose metabolism, but also the dynamics of GLP-1 in the presence of *H. pylori* and upon antibiotic therapy (23). GLP-1, mainly produced in the ileum and colon by enteroendocrine L cells, regulates glucose homeostasis by systemic effects on pancreatic β cells, reduction of gastric acid secretion, delaying gastric emptying, regulating appetite as well as adipose tissue physiology (177, 178). Taking into account that some gut bacteria modulate intestinal enteroendocrine L cell secretion of GLP-1 (122, 179), it is of interest to explore whether both *H. pylori*-induced or antibiotic treatment-induced modifications in gut microbiota could also influence GLP-1 levels. In this way, GLP-1 levels at different time point after an OGTT before and after eradication treatment in *H. pylori* positive patients as well as in *H. pylori* negative controls were evaluated. We found that changes in GLP-1 AUC after eradication therapy positively and negatively correlated with the changes in the genus *Lachnobacterium and Bifidobacterium adolescentis*, respectively. Variation in GLP-1 at 60 min after OGTT and the changes in the family *Coriobacteriaceae* also positively correlated two months after the treatment (23). Species from the genus *Lachnobacterium* and *Bifidobacterium* are able to produce SCFAs (180). Among the beneficial effects attributed to SCFAs, these metabolites have also been proposed to favor GLP-1 L cell secretion and to exert anti-inflammatory action which may have beneficial effects on glucose homeostasis and insulin sensitivity (181, 182). Studies analyzing the use of *Bifidobacterium* as probiotic reported an increase in GLP-1 production and beneficial effects on carbohydrate metabolism (183). Concordantly, type 2 diabetic patients harbored smaller

TABLE 3 | Relationships between *H. pylori* eradication therapy-induced bacterial changes and metabolic variables.

Study (Reference)	Bacterial changes associated with metabolic variables post eradication	Putative bacterial functions	Variables
Martin-Nunez et al. (10)	(↓)*Rikenellaceae* (↓)*Megamonas* (↓)*Butyricimonas*	- Acetate, propionate and butyrate production. - Ability to degrade carbohydrates.	(↑)Glucose (AUC) (↑)HbA_{1c}
Cornejo-Pareja et al. (23)	(↑)*Lachnobacterium* (↑)*B.adolescentis* (↑)*Coriobacteriaceae*	- Butyrate, acetate, and propionate production. - Ability to degrade carbohydrates. - Regulation of bile acid synthesis.	(↓)GLP-1 secretion
Martin-Nunez et al. (22)	(↓)*Delsufovibrio* (↓)*Rikenellaceae*	- LPS release - Acetate, propionate and butyrate production. - Regulation of CD36 expression	(↓)HDL levels
Yanagi et al. (24)	(↑) B:F	- SCFAs production - Bile acids - LPS release	(↓)Ghrelin levels

Summary of studies which have explored the association of the changes in gut microbiota composition after the administration of H. Pylori eradication treatment with metabolic variables. Arrows indicate the direction of the relationship between bacterial groups and clinical variables. AUC, Area Under the Curve; B:F, Bacteroidetes/Firmicutes ratio; GLP-1, glucagon-like peptide 1; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein cholesterol; LPS, lipopolysaccharide; SCFA, Short-Chain Fatty Acid.

amounts of *B. adolescentis* (184). The cause and consequences of these opposite correlations between these two groups of gut bacteria and GLP-1 secretion in *H. pylori* positive patients after eradication treatment should be further analyzed. Bacteria belonging to the family *Coriobacteriaceae*, which has been shown to be reduced in Type 2 diabetic women (185), might also indirectly favors GLP-1 secretion by generating bile acids that stimulates GLP-1 secretion in L cells (19, 186).

Relationships between microbial groups and GLP-1 levels differed between *H. pylori* infected patients and non-infected controls. Positive correlations between the genus *Megamonas* [previously related to carbohydrate metabolism (187)] and GLP-1 levels were exclusively found in the control group. By contrast, bacteria belonging to the phylum *Proteobacteria* correlated positively with GLP-1 levels only in *H. pylori*-positive patients. Notably, these correlations disappeared after the eradication treatment and shifted to *Bifidobacterium longun* and the genus *Prevotella* which correlated positively and negatively with GLP-1 levels, respectively (23).

Other authors also explored the influence of *H. pylori* eradication therapy-related gut microbial changes on ghrelin levels (24). Ghrelin is a multifunctional hormone mainly secreted by gastric mucosa that regulates body weight by stimulating appetite, growth hormone secretion, fat storage among other relevant systemic functions in energy metabolism (188). Yanagi et al., reported that changes in the *Bacteroidetes:Firmicutes* ratio were inversely related to changes in plasma ghrelin levels after the administration of triple therapy for *H. pylori* eradication (24). Paradoxically, previous studies showed that modulation of the gut microbiota by using prebiotic supplementation reduced ghrelin secretion (123).

The relationship between specific profiles of gut bacteria in *H. pylori* infected patients before and after eradication treatment and metabolism are not restricted to carbohydrate metabolism but it seems to extend to lipid metabolism. Major phyla *Bacteroidetes* (increased in *H. pylori* positive patients) and *Firmicutes* (decreased in *H. pylori* positive patients) were negatively and positively correlated with HDL/LDL ratio, respectively. When analyzed in more detail, several bacterium taxa (*Eubacterium*, *Bacteroides coprophilus*, *E. biforme*), that were increased in *H. pylori* positive patients, were related to HDL/LDL ratio (22). Specific associations with lipid profile related to eradication treatment-induced gut microbial modifications were also found. Positive and negative changes in *Delsufovibrio* and *Rikenellaceae*, respectively, predicted changes in HDL-C levels at month 2 after completing antibiotic treatment (22). *Delsufovibrio* is a producer of LPS (189), but its products can also up-regulate the expression of the critical regulator of lipid absorption, CD36 (190), that has been positively associated with HDL-C levels (191). On the other hand, *Rikenellaceae* produces acetate that, besides the beneficial SCFA effects, has been described to promote hepatic *De novo* lipogenesis and cholesterol synthesis (192).

All in all, this emerging evidence suggest that gut microbiota shifts induced by *H. pylori* and upon antibiotic treatment for its eradication might, at least in part, underlie modulation of metabolic variables. However, further studies should be performed to confirm this hypothesis and to fill the many gaps of this intricate cross-regulation. Other *H. pylori* therapies than triple therapy should be assessed, as well as the concomitant effect of pre- or probiotic supplementation during eradication treatment. In addition, the precise mechanisms and bacterial activities involved in the *H. pylori*-gut microbiota-metabolism crosstalk remains to be elucidated.

DISCUSSION

While the number of studies analyzing the effect of *H. pylori* eradication therapies on the gut microbiota are increasing in recent years, current evidence is not enough to draw clear conclusions on the most relevant and common bacterial shifts. Future research needs to face several challenges to make clear assumptions on this relationship. Different population characteristics can lead to divergent results. Gut microbiota composition varies among ethnic groups, likely due to the different dietary, hygienic and genetic factors as well as regional antibiotic resistance rates (142). Thus, the modifications on specific bacterial taxa and the underlying related mechanisms involved in metabolic regulation could largely differs depending on the region where the study is performed. In this sense, the inclusion of non-infected healthy individuals as controls in intervention studies would give valuable information to determine the actual reversion degree to "healthy" baseline gut microbiome. Moreover, it is likely that the severity of the *H. pylori* eradication therapy impact on metabolism and adiposity in the long-term also varies depending on the age group. In that way, other antibiotic treatments than those used for *H. pylori* eradication have been shown to influence the onset of obesity when applied to early-life infants, while evidence on antibiotic administration effect on adult metabolism is not so clear (31–34, 137, 138). The fact that most of the studies analyzing the gut microbiota in *H. pylori* infected patients excluded subjects with diabetes, obesity and cardiovascular diseases (10, 16, 17) also limits the knowledge on the concomitant management of the dysbiosis induced by *H. pylori* infection and treatment in patients with these metabolic diseases.

Other unaddressed issue is how the different intestinal bacterial patterns influenced by infection severity relates to metabolic homeostasis. Furthermore, the relationship between *H. pylori*-induced changes in gut microbiome and metabolic disorders has been scarcely explored as yet, and the effect of each therapeutic options used to treat *H. pylori* infection as well as the concomitant use of probiotics on specific metagenomic and metabolic modifications should be also further addressed.

Finally, to the best of our knowledge, direct evidence on the bacterial functions and actions (by-product production, inflammation and translocation of bacterial products) resulting from the modulation of the diverse bacterial groups as a whole during *H. pylori* infection and treatment remains unexplored. This might be helpful to improving the understanding of the metabolic regulation by gut microbiota within the context of *H. pylori*-related disease.

In conclusion, although current data points at an essential role of gut microbiota as mediator of the crosstalk between *H. pylori* and host's metabolic health, the numerous remaining unanswered questions warrant future in-depth research in this field.

AUTHOR CONTRIBUTIONS

MC-P and FJT contributed to conception and design of the manuscript. GMM-N, IC-P and MC-P researched the literature. GMM-N, IC-P, MC-P, and FJT drafted and wrote different sections of the manuscript and contributed to the discussion and interpretation of data. All authors contributed to the article and approved the submitted version.

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